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Target Recognition and Competitive Synaptogenesis in the Drosophila Giant Fiber System

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TARGET RECOGNITION AND COMPETITIVE SYNAPTOGENESIS IN THE DROSOPHILA GIANT FIBER SYSTEM

A Dissertation Presented

by

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ABSTRACT

TARGET RECOGNITION AND COMPETITIVE SYNAPTOGENESIS IN THE DROSOPHILA GIANT FIBER SYSTEM

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The development of complex neural networks relies on a careful balance of environmental cues to guide and shape both ends of the eventual connection. However, the correct wiring of circuits whose components share molecular profiles depends on a more elaborate phenomenon, competition. Despite being highly studied, there is still a lack of understanding as to the mechanism that allows molecularly identical cells to form exclusive connections with their targets. To address this complex question, we turned to a simple circuit within the genetically tractable fly. Responsible for the escape reflex, the Giant Fiber System is comprised of bilaterally symmetrical axons that innervate the ipsilateral “jump” motorneuron, TTMn in a 1:1 ratio. However, if a TTMn is unilaterally ablated prior to circuit formation, this ratio is disrupted and the deprived axon forms its presynaptic terminal on the opposite side.

Midline crossing by the deprived axon led to exploration of a known pathway in giant fiber development, midline repulsion via Slit and Roundabout. Axons in which Roundabout levels were reduced through a natural pathway antagonist,

Commissurelesss, crossed the midline freely, confirming a native, if normally restricted ability to do so. However, unlike the overlapping giant fiber terminals seen following ablation, these axons retained their wild exclusivity, elaborating their terminals toward a single TTMn. This supported our initial aim of uncovering a competitive force in giant fiber target selection.

In addition to repulsion, I also examined the attractive pathway of Netrin and Frazzled for a possible role in target identification. Varying the levels of Frazzled receptors led to increased midline crossing and overlapped terminals, suggesting a connection between this attractive receptor and the repulsion pathway first examined. Frazzled has been shown to induce *commissureless* expression independent of its ligand, making it an important linchpin in the regulation of giant fiber guidance and competition. In fact, when allowed to traverse the midline, giant fibers responded to a Netrin increase with overlapping synaptic terminals. In this dissertation, I present a model in which giant fibers possess competitive machinery, driven by Netrin and triggered by Frazzled, underneath the naturally restrictive midline repulsion.

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BACKGROUND AND SIGNIFICANCE

1.1 Wiring a Brain

1.1.1 Specific Guidance Using General Markers

The nervous systems of animals can be thought of as a highly integrated, interconnected web of circuits. The most remarkable aspect is not the complexity, however, but the fact that this web is self-assembling. Cells in a circuit must grow, navigate, and connect in a sea of similarly dynamic cells. Imagine trying to find a friend in a mall during the holidays while everyone else at the mall is simultaneously looking for someone they know. The overwhelming din of communication and faces is a perfect example of the environment in which complex circuits – circuits that will be responsible for every aspect of life – must form.

During development of a nervous system, axons must successfully navigate to, identify, and in some cases fight for their proper synaptic partners. The first stage of this, navigation, is accomplished through a tightly regulated system of stop and go signals. These guidance cues come in four major flavors: long distance attraction, long distance repulsion, contact/short range attraction, and contact/short range repulsion (Dickson & Keleman, 2000). Together, these signals act as signposts and landmarks for developing neurons. While the specific expression pattern within brains of different species may vary, the functions remain constant,

provide simple, robust route markers and provide guidance for receptive cells. Subtlety is added by the individual neurons, which may respond to the same cue in different ways. For instance, at the optic chiasm of higher vertebrates, retinal ganglion cells (RGCs) coming from one eye have a choice. The chiasm is home to radial glia cells that produce a protein of the Ephrin family, Ephrin B2 (Mason & Sretavan, 1997). Axons originating from different parts of the same retina respond differently to this cue, however. RGCs from the temporal most aspect express the matching receptor, Eph B1, and respond to the midline with repulsion. These cells do not cross the midline, but rather grow toward the ipsilateral hemisphere of the brain. This is contrasted by RGCs from the nasal most aspect of the retina, which do not express the Eph B1 receptor. These axons ignore the Ephrin B2 at the optic chiasm and readily cross the midline, growing toward the contralateral hemisphere of the brain.

Another way axons vary their interpretation of common signals is through temporal fluctuation. Varying its sensitivity to a signal allows a cell to become temporarily deaf to its influence. For example, commissural axons in the embryonic fruit fly cross the midline at specific locations. The cells grow along the midline, prevented from crossing due to their sensitivity to a protein, Slit, produced by the midline glia and bound by the surface receptor, Roundabout (Bashaw et. al, 2000; Kidd et. al., 1998). In order to cross, these cells upregulated a protein called Commissureless, whose function it is to prevent the Slit receptor, Roundabout, from reaching the surface of the axon. Now unable to respond to midline repulsive

signals, the cell grows across. Once across, Commissureless production is halted, allowing Roundabout to return to the surface – restoring Slit responsiveness (Keleman et. al., 2005; Rosenzweig & Garrity, 2002; Couch and Condron, 2002). This temporal control of signal response translates into very specific, single crossing events.

Guidance cues exist in concert, providing a near constant influx of push and pull forces to any cell that can be affected. In commissural axons, both in vertebrates and those described above in fruit flies, crossing the midline depends on both an insensitivity to Slit, but also an attraction to cues like Netrin, a secreted ligand that signals attractive growth through its receptor, Frazzled (Kidd, 2009; Garbe & Bashaw, 2007). This results in an overall force drawing the axons across the midline. However, once across, there is a shift whereby growth cones switch their receptor profiles. By shifting from an abundance of Frazzled to a profile rich in Roundabout, they simultaneously lose their attraction to molecules like Netrin and gain repulsion from molecules like Slit (Sabatier et. al., 2004; Brose et. al., 1999; Kidd et. al., 1998). This integration of multiple signals by each neuron creates a unique, dynamic, and delicately balanced response over time. When paired with the discrete landmarks, axons are able to navigate through the cluttered nervous system in shorter blocks of distance, hopping from one signal to the next to find their way to their final target (Brierley et. al., 2009; Tessier-Lavigne & Goodman, 1996).

Guidance cues and molecular profiles provide a simple, cellular mechanism for guidance to a specific point. Imagine again that crowded mall. Locating your

friend becomes a discrete course if you have a list of directions based on landmarks. While those landmarks don't change, each person can receive a different list of instructions, making the sorting simple and individual. The same is true for the developing nervous system. The signal sources are relatively constant, but the response of each population of cells is unique and specific to guide them to the appropriate target field.

1.1.2 Unique Pairing From Identical Options

The elegantly simple guidance of cells to their synaptic targets is the first step in forming a specific connection from a massive pool of options. Once to their destination, axons are then required to identify and synapse with a specific target. Target selection relies on both guidance cues along the path as well as specific molecular identities of the target cells themselves (Kasthuri 2003; Winberg et. al., 1999; Tessier-Lavigne & Goodman, 1996). However, in many neural circuits this selection is compounded by the presence of several potential pre- and postsynaptic elements. In vertebrates and many invertebrates, the visual systems (Huberman et. al., 2005; Stellwagen & Shatz, 2002; Ashley & Katz, 1994), neuromuscular junctions (Walsh & Lichtman, 2003; Winberg et. al., 1999; Chiba et. al., 1993), and even olfactory system (Luo & Flanagan, 2007; Jeffries, 2006; Marin et. al., 1995) rely on the proper sorting of several similar inputs amongst a common target field. The molecular identity that helped unite axons and dendrites now becomes a hindrance, with each axon being a viable input for each target cell. In fact, in many systems,

early development of the circuit shows several overlapping and redundant connections, with axons connecting to several targets, which in turn receive input from several axons. This web of non-specific synaptic contact demonstrates the interchangeability of the various components. But then how do specific circuits form? What mechanism exists to restore uniqueness to axons, separating them out from a ubiquitous pool? The answer – first uncovered and initially characterized in the visual cortex of kittens – is competition.

1.1.3 Competitive Synaptogenesis

The first example of a competitively sculpted neural system was observed and characterized in the late 1950's through early 1970's by David Hubel and Torsten Wiesel – for which they were awarded the 1981 Nobel Prize in Medicine, shared with Roger Sperry. Their work initially centered on an in-depth description of the visual cortex layers in postnatal cats. By focusing on cellular recordings at various depths, they were able to pair anatomical position with electrophysiological responses to various visual stimuli. Remarkably, they found that retinal cells had a level of response specificity previously unimagined. The existence of "on" and "off" response cells had been previously demonstrated by Stephen Kuffler and opened the field to the notion that there may be further specialization. In his Nobel lecture, Hubel recalled the serendipity of their initial discovery:

> We were inserting the glass slide with its black spot into the slot of the ophthalmoscope when suddenly over the audio monitor the cell went off like a machine gun. After some fussing and fiddling we found out what was happening. The response had nothing to do with the black dot. As the glass slide was inserted its edge was casting onto the retina a faint but sharp shadow, a straight dark line on a light background. That was what the cell wanted, and it wanted it, moreover, in just one narrow range of orientations. (D. Hubel, Nobel Lecture – 1981, p27).

This result was the first of many insights into the construction, function, and development of the primary visual cortex. Their research spanned a breadth of architectural deconstruction for which an appropriate summary would be unmanageable in this introduction. In an unthinkably short span of time, they uncovered columnar preferences for stimulus orientation, size, shape, and motion – greatly impacting the field of mammalian neurobiology (Hubel & Wiesel, 1962). Specifically relevant, they also uncovered an interesting bias of some cells for stimulation from a single eye. They termed this “ocular dominance” and described its presence in the visual cortex, as well as lateral geniculate nucleus (Hubel & Wiesel, 1965, 1963a, 1963b, 1962, 1961; cartooned in Figure 1.1A). They decided to explore the development of these highly specific responses from the angle of experience, aiming to explore the effect early function of the visual system could have on its own structure. In an effort to produce internal controls, they began by

monocularly depriving kittens of visual input through lid suturing. This produced a drastic effect in the striate cortex, where ocular dominance shifted greatly in favor of the unaffected eye (Wiesel & Hubel, 1965, 1963a, 1963b), while the lateral geniculate nucleus maintained a binocular response (Wiesel & Hubel, 1963a, 1963b; cartooned in Figure 1.1B, C). Upon binocular deprivation, nearly all ocular dominance was removed, demonstrated by an even response from both eyes in regions receiving input from both retina (Wiesel & Hubel, 1965; cartooned in Figure 1.1B, C). The initial expectation was for a global drop in responsiveness, consistent with the monocular result. However, the switch from a selective system to a permissive system was the first indication that a native selective pressure had been removed. Furthermore the effects of monocular and binocular deprivation (and rescue from these effects) were only seen if administered during the early stages of postnatal development, dubbed the "critical period" by Hubel and Wiesel (Wiesel & Hubel, et. al., 1970; Hubel & Wiesel, 1965a). Characterization of a "critical period" indicated that this was a developmental process, occurring as the initial connections were forming and maturing. This work eventually was extended to primates, when they uncovered the same phenomenon in the visual systems of juvenile macaques (Hubel et. al., 1977).

In their revealing of neural competition, Hubel and Wiesel also created an experimental paradigm to study it. A **competitive model** can be defined as a system in which two or more similarly sourced inputs have the potential to innervate a common target. This innervation is seen in the mature system as a strong

preference for one input, or exclusive control by a “winner”. Because competitive systems are plastic in early stages, experimental perturbation can be used to remove, silence or weaken components of the circuit, effectively letting competitive forces push the system in a new way. The resulting phenotype provides clues as to how competitive forces work in the wild type animal. This paradigm has been applied to circuits in the nervous systems of several model systems, each with its own unique traits. While each has provided more insight into the phenomenon in general, fundamental understanding of how seemingly indistinguishable components arrange into specific and unique circuits is still lacking.

1.2 Competitive Models

1.2.1 Competition Studies in Vertebrates

The visual cortex of cats continues to be utilized in studying neuronal competition (Lein et. al., 1999). The homologous studies are also being explored in macaque (Keliris et. al., 2010), rat (Cerri et. al., 2010), and mice (Morishita et. al., 2010). The early deprivation studies led to the discovery that the relative activity level of retinal axons, in the forms of spontaneous waves, is crucial to their ability to compete (Katz &Shatz, 1996). This refinement of the model led to use of activity blockade in place of lid suturing in some studies, allowing for a more specific disruption of cell populations (Lein et. al., 1999). This blockade technique has lead to the identification of the importance of Brain Derived Neurotrophic Factor (BDNF)

in synaptic competition. BDNF has been shown to link retinal activity to the survival/strengthening of synaptic components. BDNF has also been implicated in retrograde signaling of Nerve Growth Factor (NGF) to bias neural survival in circuit formation (Sharma et. al., 2010). Additionally it has been found to promote hippocampal long-term potentiation (LTP) through its receptor, p75 in mice (Woo et. al., 2005). In the cat visual system, it appears that BDNF levels at the subplate of the cortex are able to affect the activity levels of incoming axons from the Lateral Geniculate Nucleus (Lein et. al., 1999). This last result was uncovered by chemical ablation of these subplate neurons, once again demonstrating the usefulness of the removal paradigm described earlier. Additional work in the mammalian Lateral Geniculate Nucleus (LGN) has shed some light on molecular underpinnings of competition. Though Roger Sperry shared the Nobel Prize in 1981 for his studies with cerebral hemisphere separation, his seminal characterizations of vertebrate visual systems introduced the concept that a neuron's topographic origin within a retina could influence the location of its innervation of the LGN, Superior Colliculus (SC), and visual striate (Sperry, 1963). This positional mapping phenomenon was later discovered to be due to gradients of matched receptor and ligand pairs in the retinal ganglion cells (RGCs) and LGN, respectively. In brief, a cell's origin within the retina was correlated with a relative amount of an ephrin receptor (Eph family) with different receptor gradients assigned to different axes (Lemke & Reber, 2005). Additionally, the synaptic target fields of both the LGN and SC established matching gradients of the appropriate ephrin ligands. The relative amount of each receptor a

cell had was a function of its location within the retinal map; simultaneously dictating the position to which it would be guided (Lemke & Reber, 2005; Reber et. al., 2004). The first binocular mappings of the LGN were done in the mid 1950's and showed distinct regions representing the retinal projections from each eye (Hubel & Wiesel, 1962a). These broad regional restrictions were also found to be a function of Eph/Ephrin mapping, with the borders between regions directly affected by over-expression in the retina (Huberman et. al., 2005). However, these mapping studies do not address competition between projections, simply their ability to refine borders based on a receptor/ligand grid system. What remains controversial in these experiments is the similarity between axons at these borders. While they do represent regions of the retinal stimulated by the same sources of light (Lemke & Reber, 2005), they do not originate from the same topographic region on each retina, making them potentially unique, especially in regards to mapping receptor proteins like the Eph family.

Another popular model system used to study competitive synaptogenesis is the vertebrate neuromuscular junction. During development, these synapses undergo a process known as synapse elimination (cartooned in Figure 1.2). In prenatal stages, each muscle fiber receives overlapping input from several motorneurons at several sites of clustered postsynaptic receptors (Figure 1.2A). These overlapped connections are refined through development, with the presynaptic terminal of one motorneuron persisting and strengthening and the other retreating. This competition results in the adult innervation pattern where each

muscle fiber receives input from a single motorneuron (Buffelli et. al., 2003; Walsh & Lichtman, 2003; cartooned in Figure 1.2B). Interestingly, there is no correlation between the initial sizes of each competitor's terminal and the eventual outcome. Similarly, the coverage ratios shift randomly during competition, making it impossible to predict the winner before elimination is complete (Walsh & Lichtman, 2003). However, globally silencing the competitors and selectively exciting an individual axon produced a predictable winner, supporting the idea that the relative activity of the competitors affects their ability to compete, similar to the visual system (Buffelli et. al., 2003). This effect has also been replicated in vitro. Co-culture of motorneurons with isolated muscle fiber cells produces "dually-innervated spherical myocytes" which can be selectively manipulated electrophysiologically (Tao & Poo, 1994; Lo & Poo, 1991). Blockade of activity, followed by selective stimulation of one neuron leads to its successful formation of mature synapses. While these systems are being explored in finer electrophysiological detail (Garcia et. al., 2011; Garcia et. al., 2010; Favero et. al., 2009), there still exists a void in the understanding of how competition functions to distinguish two similar inputs.

The studies in vertebrate systems are extensive and elegant. They approach a dynamic, robust phenomenon with direct manipulation and subtractive precision. While the complexity of vertebrate models makes them highly relevant to understanding human neuronal development, it also makes analysis highly convoluted. As with many complex questions, a simpler approach may benefit the process.

1.2.2 Competition Studies in Invertebrates

Synaptic competition has also been widely studied in invertebrates. Model organisms that were once thought to be genetically hardwired and incapable of plastic synaptogenesis have been shown to rely heavily on competitive interactions to guide, establish, and refine connections. The flexibility and simplicity of these systems has been a great aid in probing competition. Invertebrate systems have shown this process to be wide reaching – observed in peripheral as well as central nervous systems; manifested not only in axons but dendrites as well. Establishing the proper connections within a nervous system requires the molding of not only presynaptic, but also postsynaptic cells. In addition, invertebrates have been used to study the role of presynaptic activity in synaptic competition, similar to work from vertebrate systems, making them a useful tool in the search for the underlying mechanism of competitive synaptogenesis.

1.2.2.1 The Myth of the Inflexible Invertebrate

Historically, the invertebrate nervous system was believed to be entirely wired based on an inflexible genetic blueprint and lacking competition, due in part to its highly stereotyped circuitry (Easter et. al., 1985). However, this was countered with work in the cricket sensory system (Murphey, 1986a, 1986b) that showed an inherent plasticity to that system. Since then, work spanning several invertebrate model systems has revealed the presence of competition in the development of pre-

and postsynaptic elements. There still exists, however, a sense in some literature that much of the nervous system is formed following an entirely preset schematic. This is a comfortable model, since traditional guidance plays a large role in such basic nervous systems. In their 1999 paper, Winberg et. al. described the role of guidance molecules in arranging the motorneuron connections within a hemisegment of larval *Drosophila* (Winberg et. al., 1998). Through specific and global alterations in canonical guidance molecule expression levels, they show that the chemical profile of a target muscle fiber is a major determinant of which motorneurons innervate it (Winberg et. al., 1998). The conclusion is that specific targeting and innervation exclusivity is solely a function of molecular coding, a trait controlled almost entirely by genetic pre-programming. This work followed experiments in the fly's larval neuromuscular junctions that showed removal of a muscle fiber resulted in its synaptic partner forming ectopic connections on another fiber (Cash et. al., 1992), a result interpreted as anti-competitive. However, this last group reversed their characterization of the fly NMJ four years later when they showed territory invasion by neighboring axons following denervation or activity blockade (Chang & Keshishian, 1996).

The role of competition in larval fly hemisegmental innervation patterns has been highly debated. Research that had been interpreted as competition was later adjusted to non-competitive in light of other studies and vice versa. However, the claim that work in single hemisegment shows an absence of competition is based on a false definition of competition. *Drosophila*, like other invertebrates, have bilaterally

symmetric, simple circuits, meaning innervation at the larval NMJ is made by a single motorneuron, unique to that side, but mirrored across the midline and echoed in every segment. The well-characterized pattern of muscles and the motorneurons that innervate them is one comprised of non-homologous cells, with unique molecular profiles (Winberg et. al., 1998). Remember in the definition pulled from other competition systems, the first constraint when looking at potential competition is that it be between homologous cells – those of a common background and molecular identity. Cash et. al. deleted a target muscle (5) and saw the native synaptic partner jump to another muscle fiber (12), despite the fact that the two muscles have different molecular profiles and are normally contacted by non-homologous axons (Cash et. al., 1992). To properly use the larval NMJ as a competitive model, we would have to look at a motorneuron from one hemisegment interactions with its homolog across the midline in the other half of the animal. Invertebrate systems that account for this guideline are better suited to examine the same competition phenomenon established in vertebrate systems.

1.2.2.2 Axo-axonal Competition

As axons grow, filipodia extend and retract from the active zone of the growth cone, sampling the extracellular matrix and neighboring cells. These processes provide the cell with attractive and repulsive signals from the environment that guide the direction and speed of growth. This input is not only used to identify potential synaptic targets, but also possible competitors. Two axons growing along similar

tracts are in contact with each other via these transient processes, and have been shown to use this to affect one another. During the development of retinotopic maps in the fruit fly visual system, R7 axons in neighboring ommatidia cassettes have been shown to have a very pronounced mutually repulsive effect (Ashley & Katz, 1994). Axons growing adjacent to one another provide opposing forces that each use to establish growth boundaries (cartooned in Figure 1.3A, panel I). These boundaries eliminate invasion of an axon into the intended synaptic region of another. However, if this system is perturbed by the removal of a neighbor or addition of axons to one cassette, we find that axons response to the crowding with peripheral invasion, and do so with high regularity (Figure 1.3A, panel I – red cell; Ashley & Katz, 1994)

Competitive interactions between axons have also been shown to play a role in the degree of axon arborization. In the cricket, sensory axons with bilateral arbors were seen to shift branch complexity toward a side, which had undergone elimination of other inputs to that region [cartooned in Figure 1.3A, panel II; Murphey et. al., 1984). This shift toward one side came at the expense of the other side's synaptic efficiency, suggesting that the axon was limited in total possible synapses formed and able to alter the distribution of its branches in response to a change in the competitive environment (Sheppard & Murphey, 1986). Again these results show that axons are capable of establishing connections in these regions, but are normally kept from doing so by interactions with homolog afferents.

1.2.2.3 Dendro-dendritic Competition

Competitive interactions are not limited to axons in invertebrate nervous systems. Dendrites are often found in comprehensive but non-overlapping fields, a phenomenon known as dendritic tiling. This competitive interaction between homologous neurons ensures that dendrites receiving similar input fill an entire region without redundancy. The first requirement that makes tiling possible is the issue of self and homolog recognition. It has been shown in the leech that branches of large dendritic trees will avoid overlap with other branches of the same cell. However, if a branch is severed from the tree, there is a loss of identity and the two disconnected parts will overlap extensively (Wang & Macagno, 1998; cartooned in Figure 1.3B, panel I). This loss of self-avoidance suggests that the inhibitory effect that normally prevents single cell redundancy requires a connection of the cell membrane, perhaps through a shared cell surface protein. The phenomenon is also seen in the PNS of embryonic fruit flies where dendrites extend from bilaterally symmetric multiple dendritic (md, a.k.a dendritic arborization, da neurons) neurons. Each neuron elaborates a dendritic field without fasciculation or overlap with itself, identical to the sensory arbors described above (Corty et. al., 2009; Millard & Zipursky, 2008; Grueber et. al., 2003). Recent work demonstrated that self-recognition and avoidance are controlled by a protein known as Down Syndrome Cell Adhesion Molecule (Dscam). This member of the immunoglobulin superfamily mediates a homophilic-binding repulsion via three Ig-like domains (Hattori et. al., 2008; Matthews et. al., 2007). Remarkably, the Dscam locus uses alternative

splicing of each domain to encode more than 38,000 alternatively spliced isoforms (12 variants of I, 48 variants of II, and 33 variants of III =19,008 unique extracellular domains paired with 2 possible intracellular domains). It was proposed that these isoforms are arranged in heterogeneous multiplets on the surface of competing cells (Hattori et. al., 2007). This enormous number of possible combinations means a single gene is capable of producing a unique protein profile for nearly all neurons in the fly (Hattori et. al., 2009; Millard & Zipursky, 2008). Dscam has been found to play a role in other networks, such as the Mushroom Body (MB) of flies (Jeffries et. al., 2006; Jeffries, 2004) and in layer specification in chick retinal ganglia (Hattori et. al., 2008), though it does not undergo as extensive alternative splicing in mammalian systems. This may alter its functional role, given that its usefulness in flies was seen to depend on at least 4,000 isoforms being present (Hattori et. al., 2009).

The Dscam family was originally investigated for a role in tiling of neurons. Tiling refers to the complete, but not redundant coverage of a receptive field by several dendritic arrays of a certain class. This boundary is established and maintained via competition between members of a class (Grueber et. al., 2003). Unlike self-recognition, the large variety of surface profiles provided by Dscam isn't required here. Rather, a class-specific signal is required to allow neurons to meet and form appropriate borders. While a family member, Dscam2, has been implicated in the "tiling" of retinal columns in the fly medulla (Millard & Zipursky, 2008), no direct evidence exists that Dscam is involved in dendritic tiling. However, research has pointed toward a few possible candidates. Unilateral ablation of a

Class IV ad neuron, caused dendrites of its homolog to grow past the midline into the territory normally occupied by the missing dendrites (Gao et. al., 2000). This particular process has been shown to be dependent on the protein Flamingo (Fmi; starry night), a member of the protocadherin subclass. This membrane-associated protein is thought to mediate the interaction via homophilic binding (Kimura et. al., 2006; Gao et. al., 2000). Dendritic fields are thought to establish borders with this mutual repulsion (cartooned in Figure 1.3B, panel II). However, this result appears unique to class IV neurons, despite an adhesion role in the early development of all classes (Kimura et. al., 2006; Sweeney & Davis, 2002; Gao et. al., 2000). It has been suggested that each class may, in fact, rely on a unique cue to establish its mutual repulsion. In fact, several possible mediators have been identified and proposed to fill this type of role, including Celsr2 and 3 (Shima et. al., 2007) Shrub, Turtle (reviewed in Corty et. al., 2009) Furry, Hippo, and Tricornered, Warts and Salvador (Emoto et. al., 2006, 2004). These studies together further the idea that competitive interactions rely heavily on surface protein expression to mediate recognition and possibly sorting of competitors.

1.2.2.4 Axo-dendritic Interactions

The growth and eventual connection between dendrites and axons is a carefully balanced interplay between attractive and repulsive forces. While repulsive forces are used to maintain borders and refine connections, attractive forces provide the drive for these competitive processes. If axons couldn't detect synaptic targets,

presumably there would be no competition for them. This counterbalance of attractive and repulsive forces has been seen when a competitive system is disturbed. In the cricket auditory system, unilateral deafening causes dendrites which normally receive input from the lost afferents to invade their homolog's territory and establish synaptic connections across the midline (Hoy et. al., 1985). Interestingly, the regeneration of the eliminated sensory input does not result in the refinement of these invading dendrites (Pallas & Hoy, 1986; cartooned in Figure 1.3C, panel I). These results demonstrate two important ideas. The first is echoed in work from vertebrate systems – once a circuit is formed, competition no longer sways the development. Competition is most often seen during early development, whether it is pupal remodeling or experience-driven remodeling of the visual cortex (Ashley & Katz, 1994; Hubel & Wiesel, 1965). Secondly, synaptic elements appear to be sensitive to trans-synaptic alteration. This means that not only are competitive systems sculpted by simultaneous presynaptic and postsynaptic forces, but also these processes may not be as separate as previously thought.

1.2.2.5 Activity Dependent Competition

Neuronal activity has been shown to play a role in the guidance, target selection, and maturation of synapses in invertebrates. During the development of neuromuscular connections, presynaptic activity has been correlated with proper target selection. In fruit flies, exogenous expression of an outward rectifying potassium channel, EKO, has been used to reduce the input resistance of

presynaptic axons, making it harder for them to generate action potentials (White et. al., 2001). When applied to embryonic motorneurons, axons were shown to extend ectopic branches to non-native target muscles (White et. al., 2002). Similar activity-reducing mutations in sodium channels have also been shown to increase axonal branching in larval neuromuscular systems (Jarecki & Keshishian, 1995). However, these artificial reductions in activity have revealed a possible synaptic homeostasis monitoring system in larval neuromuscular systems. Expression of an inward rectifying potassium channel, K_{ir}, was used to inhibit muscle EPSP's in response to action potentials from motorneurons. The channel hyperpolarized the muscle and lowered the input resistance, effectively increasing the amount of current needed to generate a muscle depolarization near that of wild type (Paradis et. al., 2001). Presynaptic monitoring of postsynaptic response resulted in an increase of presynaptic release of synaptic vesicles, compensating for the reduced excitability of the muscle. This adjustment is similar to that seen in coordinated growth of muscle and neurons during development. Increases in muscle size results in lower postsynaptic input resistances, which cue presynaptic growth and synaptic output.

Competition between axons has been seen to be dependent, in some situations, on presynaptic activity. In the locust, a particular set of sensory neurons extends axons that undergo refinement during development. Bilaterally branching axons in the juvenile are pruned to an entirely contralateral arbor in adults (cartooned in Figure 1.3C, panel II). However, when the sensory input is unilaterally eliminated during development, the axon on the silenced side retains its juvenile

morphology into adulthood, extending bilateral branches (Pfluger et. al., 1994). However, when a more global blockade of sensory input is applied, both the treated and untreated sides exhibit an inability to properly prune their arbors. This shows that activity levels play an important role in the ability of axons to interact with neighboring afferents during maturation and refinement of circuits within invertebrates. This result echoes the studies in vertebrate visual systems. However, the two differ in the way they illustrate competition's dependence on activity. In the case of vertebrates, monocular activity blockade limits the ability of the deprived axons to compete, resulting in the decrease of target territory they occupy – a reduction in expansion. In locusts, a partial activity block leads to a similar inability of the blocked neurons to compete, but it manifests in reduced pruning – a reduction in retraction. This shows that, while both systems depend on activity levels to form mature connections, the specific role can differ.

1.3 A New Model System

To review, competitive systems are made up of seemingly identical players that determine a "winner" and a "loser" through an unknown process. This leads to the obvious conclusion that either this victory is random and arbitrary or can be biased in some way to favor one player and/or disadvantage another. But how is this bias created? Which players in the circuit, if any, apply this asymmetric pressure? How is it applied; what is the molecular process? The search for

competitive pressure has led to exploration of possible ways a system could bias itself toward one competitor. Unfortunately, the underlying process of competition still remains unknown. The more it is researched, though, the more pervasive it appears – stretching across nearly all model systems. While some neurons play on a field of activity-dependence, others are fighting for molecular locks and keys. But there are commonalities too. Every example of competition occurs between cells that are of a common background – bilateral homologs, topographic neighbors, ganglion cells from the same retina, and motorneurons from a shared progenitor. The rules – laid out by the literature – dictate that a competitive system, by its nature, exists between cells that are, in all other ways, indistinguishable. It would be easiest to examine cells of this description in a simple, highly tractable model system. Here we examine the circuit behind the escape reflex of *Drosophila melanogaster*, the Giant Fiber System (Figure 1.4).

The Giant Fiber System is a simple circuit, activated by a dark stimulus, and with two terminal motor outputs – the extension of the mesothoracic leg for an escape jump and initial downbeat of the wings for flight (Tanouye & Wyman, 1980). The approach of a predator causes an increased shadow and this drop in light acts as the trigger. The giant fibers are bilaterally homologous interneurons that receive primarily visual input from the lobula columnar neurons (Col A), in the form of both electrical (gap junctions) and chemical (Dα7 nicotinic acetylcholine receptor (Fayyazuddin et. al., 2006)) elements. Mechanosensory input (electrical and cholinergic) from the antennae comes in the form of monosynaptic connections on

the giant fiber dendrites (Yasuyama & Salvaterra, 1999). It is believed that the escape circuit is primarily activated via electrical rather than chemical synapses (Strausfeld & Bassemir, 1983). The giant fiber axons descend along the midline from the brain to the second thoracic neuromere where it makes two connections (Figure 1.4B). The first is monosynaptic to the medial dendrites of the ipsilateral tergotrochanteral motorneuron (TTMn). This connection is comprised of both electrical (gap junctions) and chemical (acetylcholine) elements (Phelan et. al., 2008; Allen & Murphey, 2007; Figure 1.4B, C) as the giant fiber forms a stereotyped lateral bend away from the midline along the dendrites. The TTMns are bilaterally homologous motorneurons whose axons exit the nervous system via the ipsilateral posterior dorsal mesothoracic nerve (PDMN) and innervates the tergotrochanter muscle (Tanouye 1980). This muscle is responsible for extension of the middle leg of the fly – causing the "jump" component of escape – as well as the raising of the wings in preparation for flight. The second connection made by the giant fiber is to the ipsilateral Peripherally Synapsing Interneuron (PSI), which crosses the neural midline and synapses on five dorsolongitudinal motorneurons, DLMns. These motorneurons, in turn, innervate the six dorsolongitudinal muscle (DLM) fibers, which are the major wing depressors (Allen et. al., 1998, Tanouye & Wyman, 1980). This causes the first downbeat of the wings, and is initiation of the "flight" component of escape. The addition of the PSI to the circuit acts as a delay, causing the "flight" behavior to begin after the "jump" has completed. The circuit is highly stereotyped anatomically and electrophysiologically (Allen & Murphey, 2007; Hammond &

O'Shea, 2007; Godenschwege et. al., 2002a; Jacobs et. al., 2000; Blagburn et. al., 1999; Allen et. al., 1998; Phelan et. al., 1996; Trimarchi & Schneiderman, 1993; Tanouye & Wyman, 1980) and has been a useful model system in studying behavior (Card & Dickson, 2008a, 2008b), synaptogenesis (Godenschwege et. al., 2002, 2006), axonal trafficking (Murphey et. al., 2003), synapse maintenance (Murphey & Godenschwege, 2002), and even oxidative stress (Dawson-Sculley, unpublished work). In the wild type animal, each giant fiber descends on its side of origin and forms connections to the PSI/DLM and TTMn on that side of the midline. The TTMn innervates the muscle of the ipsilateral side, while the PSI and DLMn are responsible for providing input to the contralateral set of DLMs. The stereotyped GF:TTMn target selection is due in part to a midline barrier set up using the Slit/Roundabout repulsion pathway. Midline glia secrete Slit, which is bound by Roundabout 1 (Robo1) receptors on giant fiber axons (Godenschwege et. al., 2002b). The activated receptor results in a signal that prevents the giant fiber from growing across the Slit-secreting midline.

The Giant Fiber System has the potential to be a model system well suited to studying competition. First, it is a circuit comprised of homologs on both sides of the synapse. The giant fibers descend near enough to the midline that contact is not physically impeded. The TTMn dendrites meet at the midline and form a non-redundant postsynaptic field, as seen in sensory ad neurons. Additionally, the wild type exhibits a stereotyped connection pattern of "left to left" and "right to right", perhaps indicating a pre-existing synaptogenic order. Of course, this specificity may

be dictated by the same hardwired genetics once thought to sculpt all of invertebrate nervous systems. However, the system does not need to rely on competition in nature for the model to work. If competitive forces are applied or removed, we may be able to reveal an underlying plasticity. Through the experiments laid out in the following chapters, I show that this is the case. The Giant Fiber System has a reliable boundary at the midline provided by repulsion and may not need much more to ensure proper wiring. However, there exists a second compensatory mechanism underneath simple midline repulsion that I believe is a good model system for the study of competition. Invertebrate systems have historically been used as a workbench for more complicated systems and the genetic and technical tools available when working with *Drosophila* are a great advantage to any question. Tissue-specific driver, balance chromosomes, and a near complete catalog of over-expression and loss of function mutants greatly ease molecular manipulation of circuits. In this dissertation I utilize the paradigms established by the early studies to lay the groundwork for studying the phenomenon of synaptic competition within the escape circuit of fruit flies, the Giant Fiber System.

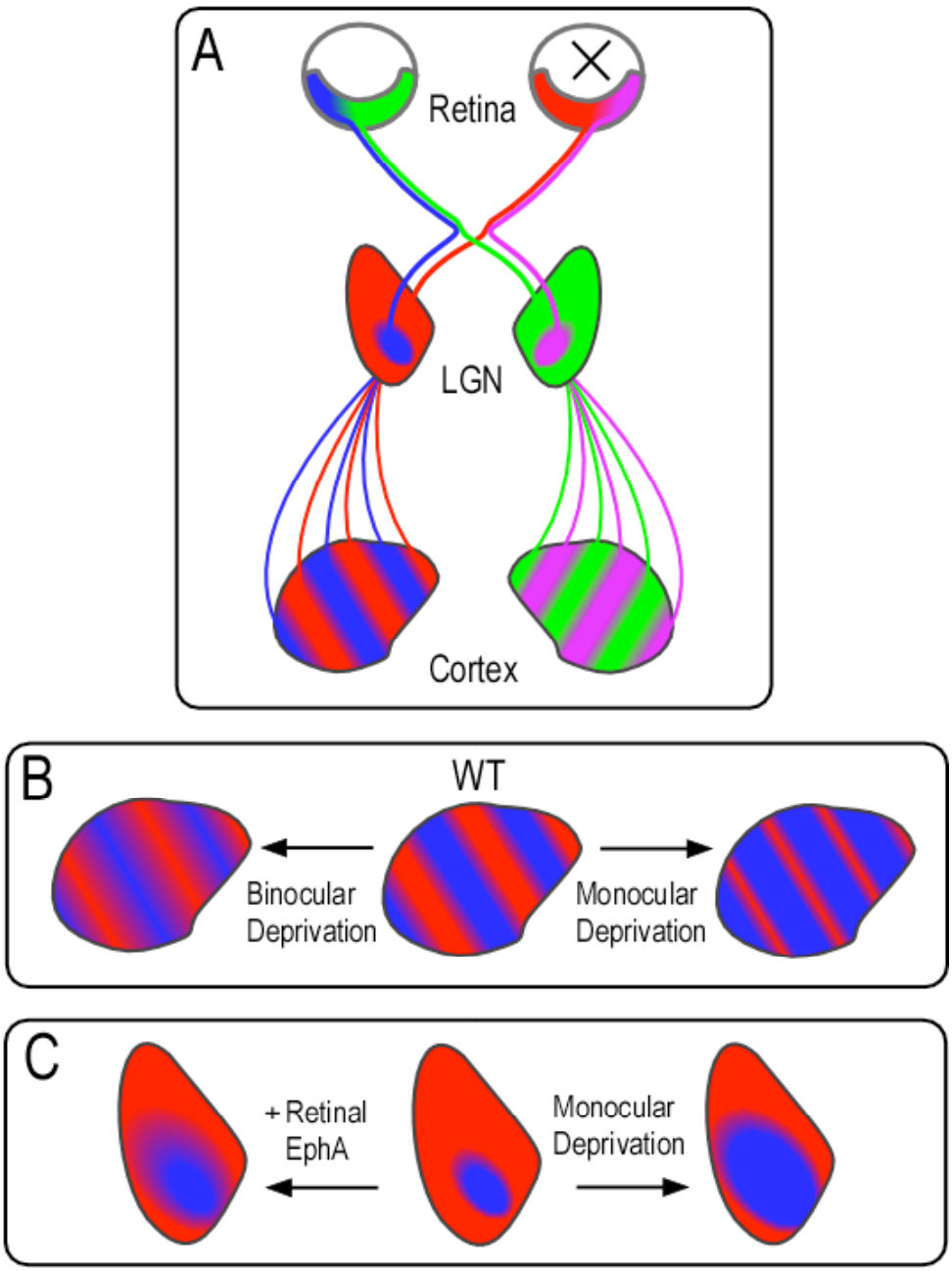


Figure 1.1. Ocular dominance and the effects of deprivation in the mammalian visual system. (A) Retinal ganglion cells from the temporal most region of an eye project ipsilaterally to the Lateral Geniculate Nucleus (LGN), where they are joined by projections from the nasal most region of the contralateral eye and synapse in stereotypic patterns. Projections from each LGN form the optic radiation tract to the visual cortex. At the cortex, bands of neurons demonstrate bias in their response to eye-specific stimuli, known as ocular dominance. (B) Ocular dominance can be affected by monocular deprivation (loss of territory by the deprived eye) or binocular deprivation (lack of refinement between columns). (C) At the LGN, monocular deprivation leads to an expansion of the non-deprived eye's territory, while alterations in Eph receptor levels in the retina blur territory borders.

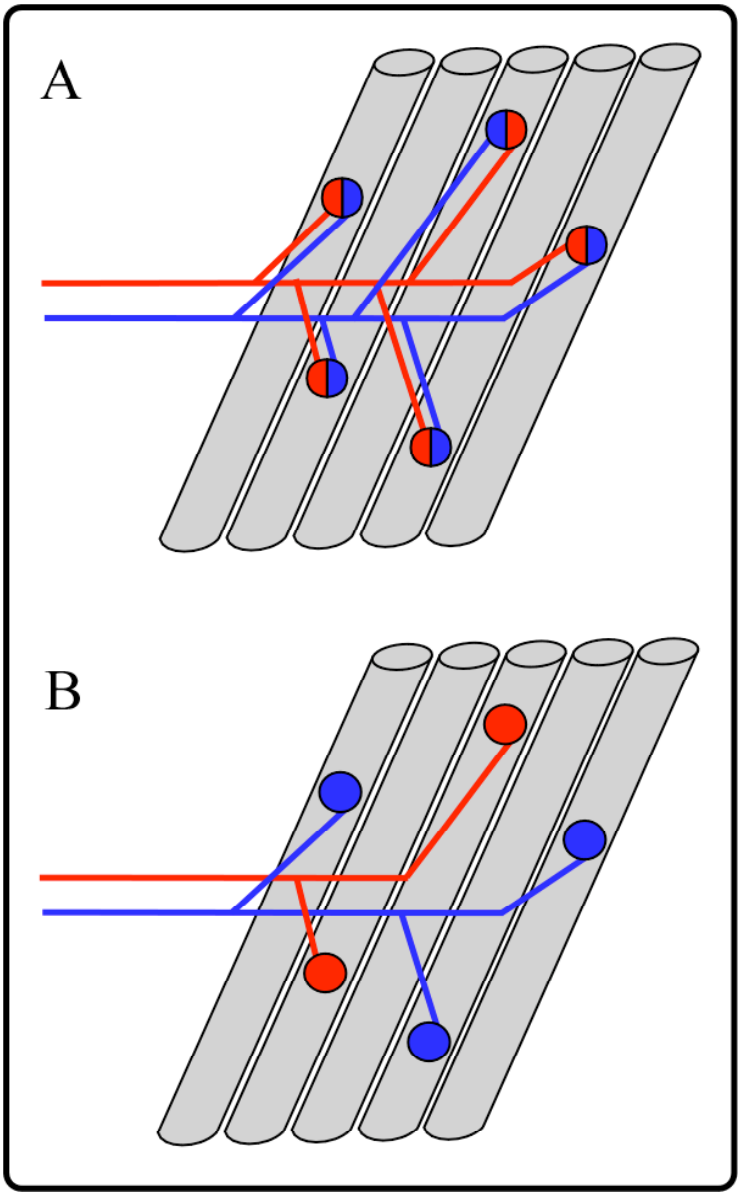


Figure 1.2. Synaptic elimination at the vertebrate neuromuscular system. (A) Early innervation at the juvenile muscle is characterized by overlapping of immature synaptic terminals from two similar motorneurons. Through development, these sites undergo competition resulting in “synaptic elimination”. The mature system has a single occupant at each site on a muscle fiber (B).

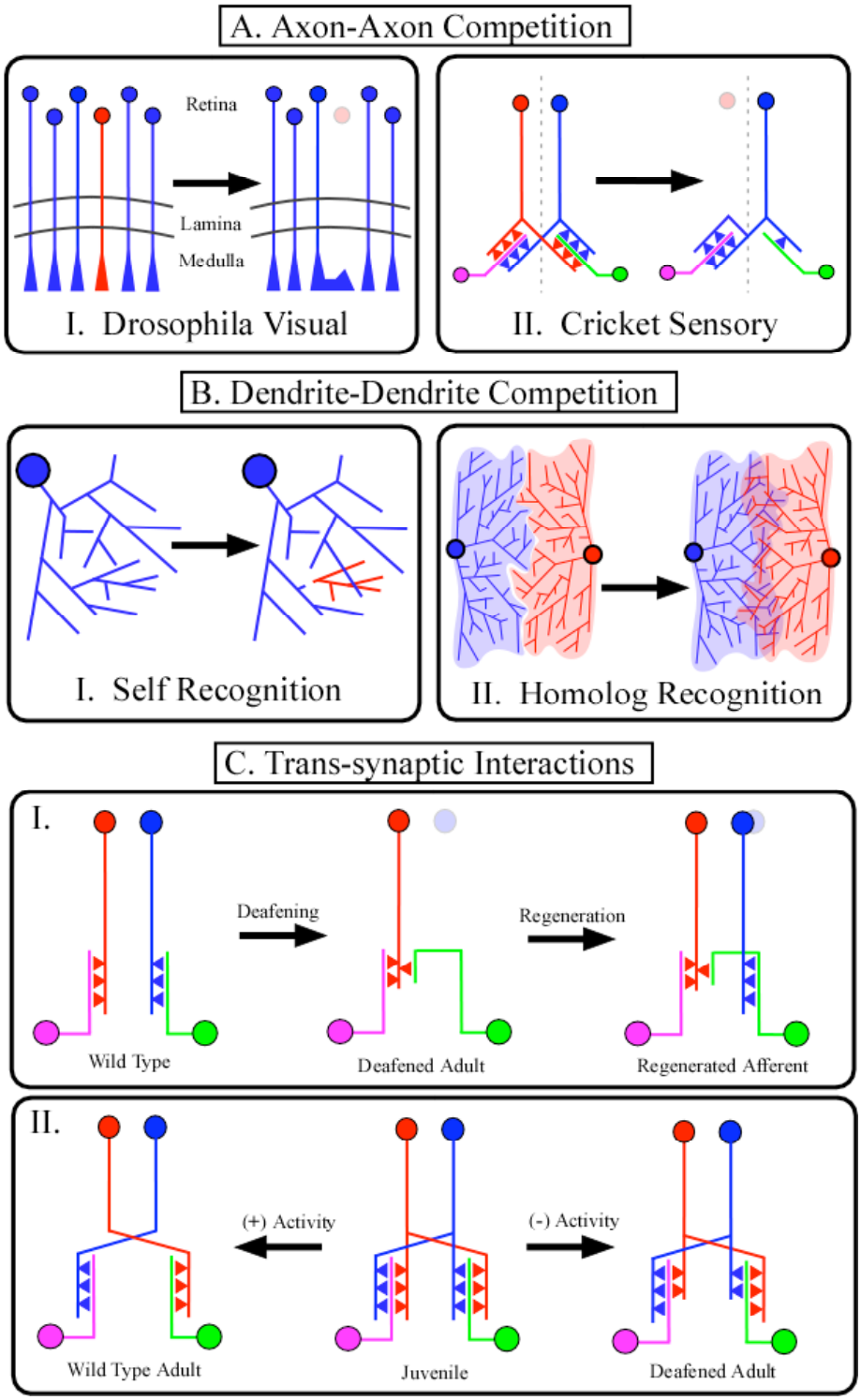


Figure 1.3. Illustrated examples of Invertebrate Competition. A1) Ting et. al., 2007 A2) Murphey & Lemere, 1984; Sheppard & Murphey, 1986 B1)Wang & Macagno, 1998; Matthews et. al., 2007 B2) Gao et. al., 2003; Kimura et. al., 2006; Grueber et. al., 2003 C1)Pfluger et. al., 1994 C2)Hoy et. al.,1985; Pallas & Hoy, 1986. In circuitry examples, pre-synaptic is at the top; postsynaptic is at the bottom of the frame.

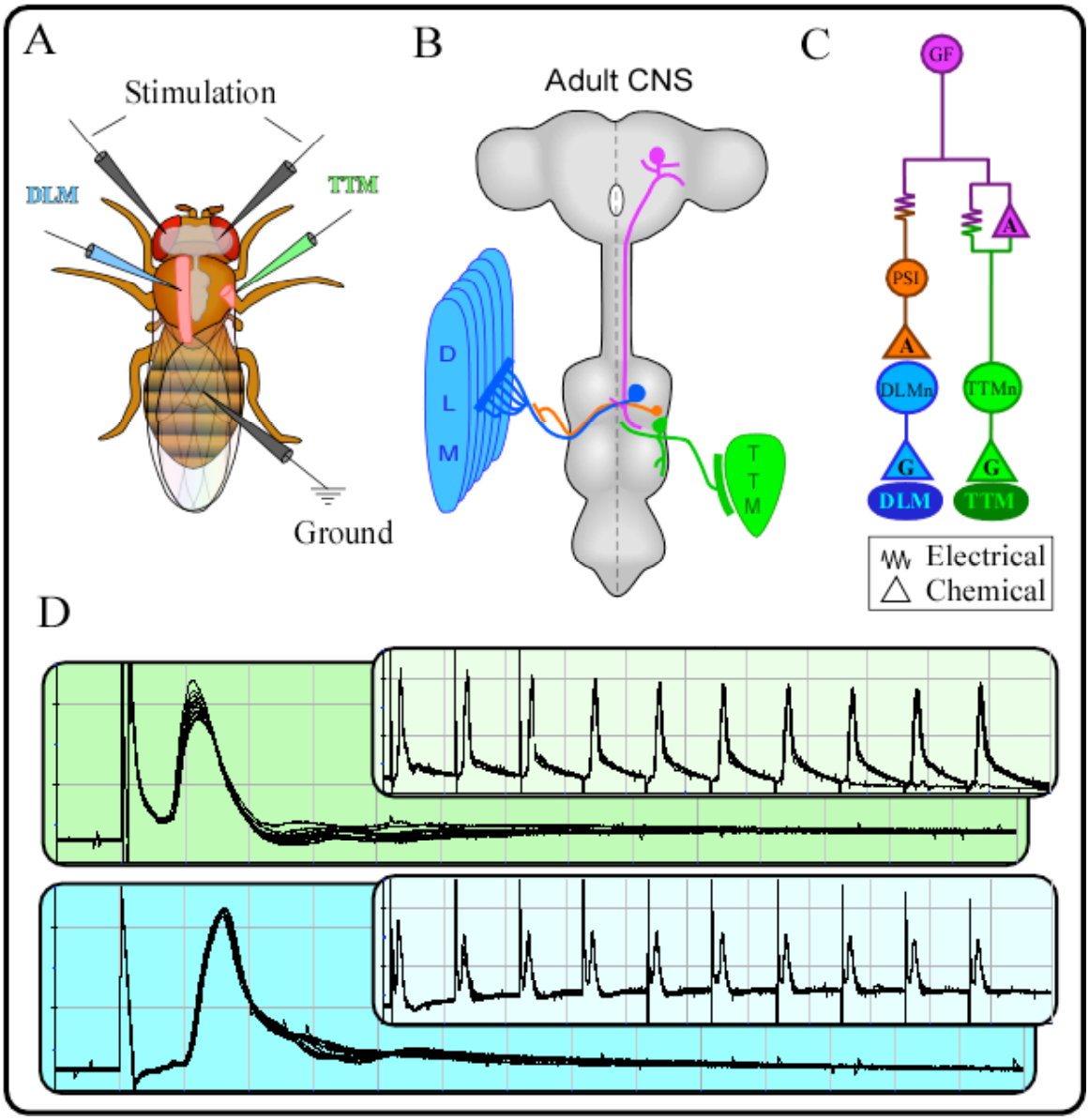


Figure 1.4. The Giant Fiber Circuit in *Drososphila melanogaster*. A) Electrophysiological characterization of the giant fiber circuit is accomplished through the global stimulation of the brain and sharp electrode recording from the "flight" and "jump" muscles, colored blue and green respectively. B) Each of the bilaterally homologous giant fiber extends from the brain, through the neck connective to the second thoracic neuromere, where it connects to both the TTMn and DLM, the latter via the PSI. C) Circuit diagram of the GFS. The GF stimulates two motor outputs: the "jump" muscle, via an electrical and chemical synapse to the TTMn, and the "flight" muscle, via an electrical connection to the PSI, which in turn synapses chemically with the DLMn. The neurotransmitter of the described chemical synapses is denoted by "A" for acetylcholine or "G" for glutamate. D) Representative latency traces from TTM and DLM following direct brain stimulation (green and blue, respectively; Major demarcations in X=1ms, Y=40mV). Insets show responses following a 100Hz train stimulation (Major demarcations in X=10ms, Y=40mV).

CHAPTER 2

ABLATION OF THE POSTSYNAPTIC MOTORNUERON(s): RESPONSE OF THE PRE- AND POSTSYNAPTIC CELLS

2.1 Introduction

During the proper formation of neuronal circuits, axons must form very specific and refined connections to the proper dendrites. This occurs through three key events, the establishment of a receptive dendrite field, the recognition of the right dendrites by the right axons, and finally the refinement of connections within the population. The issue of refinement is not trivial. Within a homologous population of axons or dendrites, the individual cells share much of the same characteristics, such as origin and surface chemistry (Winberg et. al., 1998; Davis et. al., 1997; Doe & Goodman, 1985; Doe et. al., 1985; Bate et. al., 1981). This presents both solutions and problems for reliable wiring of a complex network. In terms of identifying the proper target field, molecular similarity among populations of axons or dendrites simplifies the large number of possible synaptic partners, reducing the tangle to a set of axons looking for a set of dendrites. However, the same molecular profiles that are essential establishing these sets now serve to complicate the specificity of connections. If all members of a group share a surface chemistry, that group is unique from others, but members within a group are indistinguishable. Determining which axon from a population of equal contenders will form a connection to a specific target cell is accomplished through a phenomenon known as competition, whereby

axons or dendrites of a shared ancestry (homologs) compete amongst themselves for synaptic connections but not with neighboring cells of a different background. In other words, competition exists between kin, but not kith.

Competition was first recognized in the vertebrate nervous system in the early 1960's when Hubel and Wiesel described the plasticity of the post-natal cat visual system. They established a paradigm of system perturbation where one component of the circuit is weakened, silenced, or removed during circuit development and the system is observed after maturation. This paradigm has been applied to vertebrate and invertebrate models (Oray et. al., 2004; Stellwagen & Shatz, 2002; Reber et. al., 2004; Murphey & Lemere, 1984), central and peripheral synapses (Pfeiffenberfer et. al., 2005; Huberman et. al., 2005; Kasthuri & Lichtman, 2003; Buffelli et. al., 2003; Gao et. al., 2000), and pre- and post-synaptic elements (Grueber et. al., 2003; Ashley &Katz, 1994; Pallas & Hoy, 1986), with the same results that like competes with like. The mechanism appears to be largely bimodal, with an activity-dependent component, and a molecular component. However, some identified systems completely seem to rely on the latter. While great strides have been made to characterize both components, there are still unanswered questions as to the mechanism by which two seemingly identical neurons establish "a winner and a loser".

To better study the molecular framework of competition, we've turned to a simple circuit in *Drosophila*. Invertebrate systems were once a contentious area for competition. It was long believed that the genetic hardwiring of invertebrate circuits

excluded them from the vertebrate phenomenon of competition (Easter et. al., 1985). This was countered by work in the fly, cricket, and leech that showed competitive remodeling of sensory circuits (Gao et. al., 2000; Baker et. al., 2000; Sheppard & Murphey, 1986,). While this work has been supported by current data from *C. elegans*, some studies seem to still argue against the presence of competitive machinery in invertebrates, particularly in *Drosophila*. At the pupal and larval neuromuscular junction, mapping of guidance molecule profiles has shown that connections within a hemisegment are coded for almost entirely by unique combinations of canonical guidance pathways (Winberg et. al., 1998; Chiba et. al., 1993). At first, these results suggest that axons are simply guided to their targets by following a specific blend of signals and lack any plasticity. Accordingly, this work showed that altering a target's profile effectively changes the neurons that innervate it. However, when considered in the context of the whole animal, we find these results do not refute competition, but strengthen our definition. Within a hemisegment, the individual motorneurons, as well as muscles, are not homologs. Rather, the homolog for each motorneuron or muscle is located across the midline in the other half of the segment. While this confirms that non-homologous axons do not compete for non-homologous muscles, it does not address possible interactions between cells of a shared genealogy. This question has been addressed in the NMJ of mice, where whole muscles may be molecularly unique, but individual muscle fibers are identical; thus competitively innervated by motor neurons of a shared lineage (Buffelli et. al., 2004, 2003; Kasthuri & Lichtman, 2003). In addition, a type of

competition has been characterized in the fruit fly retina, where mutual repulsion between homologous axons leads to a columnar formation (Ting et. al., 2007; Ashley & Katz 1994). Interestingly, simple removal of competitors did not reveal the competitive nature of the system. It was only after an axon was artificially crowded and its neighbor removed that researchers were able to observe lateral territory invasion. This presents an interesting idea – that competitive mechanisms may exist in invertebrates, flies specifically, but are perhaps secondary to other, noncompetitive pathways.

The experiments presented in this chapter apply the well-established circuit dissection paradigm to the *Drosophila* Giant Fiber System (GFS), which mediates the light-off escape reflex in fruit flies. Specifically, bilaterally homologous interneurons, the giant fibers, descend along the midline to the second thoracic neuromere where they synapse with the bilaterally homologous motor neurons responsible for the "jump" response of the escape reflex, the TTMn (See Figure 1.3). This synapse is robust, having both electrical and chemical components, and is already well characterized physiologically, as discussed in the first chapter. The giant fiber system is also a simpler circuit than those studied in the *Drosophila* retina. Whereas the postsynaptic territory of those studies is an entire field and region within the brain, the target motorneurons of the GFS (TTMn) are easily identifiable as either a pair, through genetic manipulations, or individually with electrophysiology and dye injection. This presents a possible model where both sides of the synapse can be studied in depth.

Our initial approach to the Hubel and Wiesel paradigm was inspired by studies done both in cricket and frog sensory systems. Work in the frog visual system reduced the total target field size and saw an accommodation in axon growth (Reber et. al., 2004). This demonstrated that perturbation at one side of the synapse could affect the other. Deafening studies in the cricket mirrored this in an invertebrate system (Murphey, 1986; Hoy et. al.,1985). Based on this, we decided to first examine the effect of target loss on the giant fiber system. The giant fiber system forms early in pupation, with growth and contact occurring in the first 25% of development (Allen et. al., 1998; Bainbridge & Bownes, 1981). To ensure the giant fiber would develop its adult connections in a field devoid of one target, removal of the postsynaptic cell was performed in larval stages. Visualization of the postsynaptic cell was accomplished using the Gal4/UAS system to express GFP in the TTMn.

2.2 Methods

Drosophila Stocks. All stocks were raised on standard medium at 25°C unless otherwise noted. The following stocks were used: ShakB-Gal4:UAS-GFP (Jacobs et. al., 2000), hereafter referred to as ShakB:GFP.

Unilateral ablation of a TTMn cell body. Proof of concept and initial development of this technique was done with the guidance of Dr. Haig Keshishian using his facilities

at Yale University (Hartford, CT). Optimization and data collection was performed at Florida Atlantic University (Boca Raton, FL).

Late 3rd instar larvae were collected and anesthetized with diethyl ether until paralysis of the mouth hooks was observed. Any movement of the mouth hooks resulted in movement of the entire CNS, making accurate targeting impossible. The immobilized larva was then placed ventral side up in O'Dowd's saline on a non-coated slide. A 22x22mm cover slip was then used to both cover and flatten the specimen. The mounted animal was placed on a Nikon 80i microscope for cell targeting and ablation (Nikon Instruments Inc., Melville, NY). Using a 60x oil-immersion objective (Nikon Plan Apo 1.4NA, W.D. = 0.21mm), the TTMn was localized in the larval ShakB:GFP expression pattern (Figure 2.1B) and placed under pre-aligned cross hairs within the microscope optics. Maximum resolution of the objective at 23°C is roughly 192nm in XY and 509nm axially for 440nm light. The ablations were performed using a MicroPoint® Ablation Laser System (Photonic Instruments Inc., St. Charles, IL) which consists of a class IIIb nitrogen laser-pumped dye laser focused via the microscope objective. The nitrogen pump laser delivers a 2-6 ns pulse of roughly 120µJ of 337nm light; the dye, Coumarin440, produces 20-50µJ (dependent on dye age and alignment) at a max lasing wavelength of 440nm. Maximum excitation would be achieved in a volume of 0.0188 cubic microns, based on objective specifications. Effective laser power in that volume is calculated at a mean of roughly 9kW – max specification is listed at 12kW. No neutral density filtration was used. The laser was fired at 10Hz for a duration 2-4 seconds, or until

visible cell lysis had occurred. Variety in animal placement under the cover slip, as well as overall size differences resulted in an inherent variability in targeted cell depth within the animal. This depth change correlated to the overall effect the ablation laser has on the cell. More tissue between the cover slip and cell of interest led to more diffraction of the laser and less overall power at the focal volume. Hence, when targeting unilaterally, the cluster with cells closer to the surface was targeted. If close enough, the cell was visibly lysed. If too far for heat to build rapidly for lysis, the cell rapidly photo-bleached as a positive marker of laser exposure. The ablated animal was then removed from under the cover slip and placed on standard medium to recover and develop to adulthood, unless pupal dissection was specified.

Bilateral ablation of TTMn cell bodies. Removal of both jump motor neurons was achieved using methods similar to above. Ablation was performed on visible cell clusters on both sides of the second thoracic neuromere.

Electrophysiology. Adult flies were screened for positive ablations during the first 24 hours after eclosion by electrophysiologically testing the terminal giant fiber system outputs, the jump and flight muscles. In brief, sharp electrode intracellular recordings from the TTM and DLM (positive control) were collected as earlier described (Tanouye & Wyman, 1980). The physiological assay was modified and data was analyzed as previously described (Godenschwege et. al., 2002b).

Presynaptic Dye Injections. In preparation for dye injection, the central nervous system from an adult fly was dissected using standard methods and mounted ventral side up in a saline immersion on a poly-lysine coated slide. The mounted CNS was then placed under a 40x water immersion objective (Nikon Fluor 0.8NA, W.D. = 2mm) and DIC optics were used to locate the giant fiber axons within the neural connective. A glass electrode (20-80 MΩ) containing 1% aqueous Lucifer Yellow and backfilled with 3M LiCl was used to longitudinally impale the axon in the connective. The dye was injected into the giant fiber through the passage of 3-5 nA of hyperpolarizing current using a Getting 5A Amplifier (Getting Instruments, Iowa City). Images stacks were collected under widefield fluorescence using either a Spot RTse camera (Diagnostic Instruments) and MetaVue software, or a Nikon DS-Qi1 camera and NIS-Elements software. Deconvolution was performed on Z series using Autovisualize and Autoblur software (Media Cybernetics, Bethesda) or LIM 3D blind Deconvolution provided by NIS-Elements. For separate axon labeling with two colors, similar methods were used, filling one axon as described above, and the other using depolarizing current injected via a glass electrode containing 5% Rhodamine Dextran (10,000 MW) and backfilled with 1M Potassium Acetate.

Postsynaptic Dye Injection. To visualize the medial processes of a single TTMn, late pupal central nervous system was dissected and mounted as previously described. The cell body of a TTMn was visualized with GFP using the ShakB-Gal4 driver. As above, a glass electrode containing Rhodamine dextran was used to transversely

impale the TTMn cell body under a 40x water immersion objective. Depolarizing current was injected to release the dye into the motor neuron. Data acquisition and analysis was done as previously described.

Confocal Microscopy. Dissected and dye-injected CNSs were fixed for 1-2 hours in 4% paraformaldehyde, washed 3x30min in O'Dowd's saline, and cleared in serial baths of 10%, 25%, and 50% Thiodiethylene glycol (TDE). Cleared preps were mounted in a 97% solution of TDE and sealed under a raised cover slip. Where indicated, preps were alternatively cleared and mounted in similar progressions of Glycerol. Mounted preps were then placed under a 40x oil immersion objective (Nikon Plan Fluor 1.3NA, W.D. = 0.2mm) unless otherwise indicated. Confocal Z series were collected using an upright Nikon 90i microscope and C1si scan head. Collected image stacks were spectrally unmixed to resolve individual fluorophores using both Nikon EZC1 and NIS-Elements software. Early preps were imaged using a Zeiss LSM 510 Meta system mounted on an Axiovert inverted microscope and oil immersion objectives: 40x, 1.3NA; 63x, 1.4NA (Univ. of Massachusetts – Amherst).

2.3 Results

2.3.1 Ablation of a postsynaptic motorneuron

Due to its novelty, the preemptive ablation of an adult central neuron was not a technique available in the literature. Since I would be targeting an adult neuron

within a larval brain, the ablations done at the larval NMJ by the Keshishian lab served as a starting point for proof of concept (Farrell & Keshishian, 1999; Halfon et. al., 1997; Chang & Keshishian, 1996; Fernandes & Keshishian, 1996). Due to its adult expression pattern, which is primarily in the TTMns, the ShakB-Gal4 driver was used in these experiments to visualize the postsynaptic motor neuron (Figure 2.1A). Since the ablation would be performed in larvae, we first needed to locate those cell bodies in the larval expression pattern, which is more complex (Figure 2.1B). In an effort to determine the position of the TTMn neurons in the larval stage, I imaged the expression pattern of GFP under the ShakB-Gal4 driver in nervous systems ranging from late larval stage to adult (Figure 2.1C; cartooned in Figure 2.1D). Due to the rapid development in early pupal stages, specimens were dissected and imaged during the first quarter of pupation in intervals equaling 5% of development. By 25% of development, the TTMn was clearly identifiable based on its unique medial dendrite; later stages were collected less frequently at 50%, 75%, and newly eclosed adult. Each developmental stage was imaged in replicates of at least 5 nervous systems. Early stages were divided into males and females, with each set containing five replicates. Minor differences were observed, with males showing the same gross morphology and key developmental characterizations, but with slightly shorter processes, indicating either a minor delay in growth or general reduction in size comparable with their overall dimorphism.

Through all pupal stages, the TTMn was clearly recognizable due to its position in the neuropil and unique projections. In brief, at the start of pupation the

TTMn had a single neurite extended toward the midline and for the first 5% of development was nearly indistinguishable from other identified cells expressing GFP under this driver (see below). By 10% of development, the neurite of one cell in this group turned and grew back away from the midline. This diverted neurite split by 15% of development, with one process growing extensively toward the midline and the other directed caudally along the lateral edge of the CNS. At 20% of pupation, the medial branch had nearly reached the midline and elaborated a broad network of fine processes. This projection to the midline was key in identifying this cell as the TTMn. The general morphology and placement of the process within the neuromere is classical to the class of motorneuron projections (Brierley et. al., 2009; Williams et. al., 2004). Since the TTMn is the only motorneuron labeled by this Gal-4 driver with this pattern of dendrites, there are no other cells with which it could be confused following extension of this process. The lateral branch continued to grow, splitting at around 25% and continuing to elaborate through development. By 75% of development, the medial dendrite of the TTMn had mostly refined its midline processes into the primary anterior and posterior branches (Figure 2.1C, D).

For the purposes of ablation in the larval CNS, prior to the extension of the unique or directional processes, the TTMn cell body was localized to a cluster of four cells, positioned lateral and slightly anterior from a midline pair of neurons in the second thoracic neuromere. The other cells in the cluster have been proposed to be DLM motor neurons, and as such would not be involved in the giant fiber/TTMn connection (Jacobs et. al., 2000). At this stage, it was impossible to identify the

TTMn in this cluster for several reasons. First, the cells were of relatively uniform size and GFP intensity. Any difference in either was not seen to correlate to the TTMn specifically in later stages and targeting based on size or expression level yielded no effect on ablation success. Additionally, the relative position of each cell to the others was not stereotypic. Interneuron spacing and even the total number of GFP-expression neurons in the cluster (1-4) vary. To circumvent this lack of precise targeting, all cells in a cluster were targeted for ablation.

Early ablation trials proved technically challenging given an inherent variability in the equipment used. Though effective proof of concept, reproducibility was low. By incorporating a pre-centered and aligned laser, I was able to limit laser variability, reduce excitation light exposure, and optimize larval handling and anesthesia. Larval survival was an early challenge, with only 40% living to adulthood. It became apparent that minimal exposure to ether greatly increased survival rates, presenting a short time window between effective anesthetization and lethal exposure, on the order of seconds. The last seven sets of animals to be treated had a survival rate of 86.8% ± 21% (n=194). Positive ablation was confirmed by physiological examination. In positive ablations, the TTM on the ablated side showed no response to direct brain stimulation or thoracic stimulation. After adjustment of the protocol, positive ablation of TTMn was achieved in 26.3% ± 7.6% (n=205) of the animals treated. See methods for detailed procedure.

Animals with positive ablations showed a variety of physical effects. These ranged from mild (extension of the wing on the ablated side) to severe (full paralysis

of the first, second, and third legs on the affected side). Most animals exhibited the more mild phenotypes, wing extension and uncoordinated movement of the second leg on the affected side. Some animals showed no physical defects, appearing normal in locomotion and posturing.

2.3.2 Reaction of TTMn to ablation of its homolog

Physiologically, the intact TTMn was receiving enough input from one or both axons to achieve wild type response levels. Following inter-retinal stimulation of the brain, the TTM opposite the side targeted for ablation responded with wild type latencies of between 0.6 and 1.0 milliseconds and was able to follow train stimulation at 100Hz (Figure 2.2, train not shown). In contrast, the TTM on the side of targeted ablation showed no response to direct brain stimulation and did not follow a train of stimulation (Figure 2.2). A lack of muscle response to brain stimulation could be due to a disconnect at either the GF:TTMn or TTMn:TTM synapses. Thoracic stimulation is an effective confirmation of the latter connection. Stimulation electrodes were moved from the eyes to the upper crest of the thorax. Thoracic stimulation was unable to elicit a response from the TTM, confirming a defect in its innervation. The TTM on the non-ablated side responded normally to thoracic stimulation. Additionally, direct brain stimulation resulted in wild type responses from both DLMs, indicating that both giant fibers made appropriate connections to PSI, which synapsed with DLMn in turn.

Brains of adult animals identified as a positive ablation by electrophysiology were dissected and mounted for imaging of the expression of GFP under the ShakB:Gal4 driver. The ablation of the TTMn on the targeted side was confirmed with widefield fluorescence by the absence of a GFP-labeled cell on the targeted side. Negative ablations showed recovered expression in the targeted cell, supporting the characterization of a missing GFP-positive cell as confirmation of the electrophysiological results. Based on electrophysiological screening and fluorescent confirmation, the methods developed here proved an effective method to directly eliminate a single TTMn within the developing CNS of larval fruit flies, prior to pupal formation of the adult circuit.

Anatomically, there was normal morphology and positioning of the remaining TTMn cell body and its lateral dendrite. The medial dendrite, however, was extended beyond the midline with noticeable elaboration of the primary process in 94% of cases. This extension was positioned comparably to the posterior branch normally seen at the midline in the wild type TTMn medial dendrite (Figure 2.3M compared to F, solid arrowhead). Though midline crossing of this dendrite was occasionally observed in non-ablated animals, the extent of growth, thickness, and elaboration was always low (Figure 2.3F, solid arrowhead). The extended posterior branch in the ablated animal reached quite often to the center of the neuromere on the ablated side. The anterior branch of the intact medial dendrite maintained wild type morphology, superimposed with the synaptic terminal of the ipsilateral giant fiber (Figure 2.3 F, J, M – open arrowhead).

2.3.3 Response of the giant fibers to the unilateral ablation

To assess the effect of target removal, both giant fibers were examined morphologically. To avoid confusion when speaking of both giant fibers, I will refer to the giant fibers in relationship to the intact TTMn. The target-intact giant fiber will hereafter be referred to as the "ipsilateral" giant fiber, and the target-deprived giant fiber as the “contralateral” giant fiber. Once the putative ablation was confirmed by the absence of GFP expression within a dissected brain, one or both giant fibers were filled with fluorescent dyes using the described methods. The contralateral fiber was filled first with Lucifer Yellow, followed by the ipsilateral with rhodamine, except when noted. These fluorescent dyes were used to visualize the presence, direction, and morphology of the giant fiber’s lateral bend, which extends normally along the medial dendrite of the TTMn. This lateral bend is the presynaptic terminal in wild giant fibers, and is referred to as such hereafter, though specific synaptic connection between individual giant fibers and the intact TTMn remains to be characterized. To avoid confusion in describing the giant fiber, I will use the terms “bend” or “terminal” when speaking strictly of morphology. The term “contact” will be used to describe a close physical proximity between neurons, with no observable space between. Only when electrophysiological characterizations were performed will I use terms such as “connection” or “synapse” in my descriptions. These distinctions will be used throughout the remainder of this dissertation.

The ipsilateral giant fiber was not different morphologically in any measured way compared to wild type. The axon was approximately five microns wide in the neck connective, as in wild type (data not shown). The presynaptic terminal bent ipsilaterally and was of an appropriate thickness and length (Figure 2.4C-E, yellow axon). In animals where the postsynaptic GFP of the intact TTMn was photobleached during imaging, Lucifer Yellow injected into the giant fiber was seen to dye-couple following extended passage of current, indicating intact gap junctions between that fiber and the TTMn. To ensure viability of the prep for a second injection, no formal FRAP experiments were performed to quantify the frequency or rate of Lucifer Yellow migration into the photo-bleached GFP-labeled dendrites, as they would require a prolonged dye injection and imaging. Overall, with the methods available, there was no indication that the ipsilateral giant fiber had a noticeable morphological response to removal of the contralateral TTMn.

The contralateral giant fiber responded to the absence of its normal synaptic partner with reactive, directed growth. All observed contralateral giant fibers appeared to have characteristically normal morphology through the neck connective and at the PSI synapse, similar to the ipsilateral giant fiber (Figure 2.4, PSI connection marked by arrowhead). However, posterior the PSI connection, roughly half (55%) extended a single presynaptic terminal across the midline and along the region of the intact TTMn occupied by the ipsilateral giant fiber, often along the second anterior branch of the TTMn (Figure 2.4C, red axon). In these cases, the presynaptic terminal appeared normal in length and thickness. In addition to the

“crossed” axons, another 36% of deprived giant fibers terminals split and extended bilaterally (Figure 2.4D, red axon). One branch grew across the midline, along the region of the dendrite occupied by the other giant fiber and was of normal size and length. The other branch grew ipsilaterally, along the extended dendrite of the intact TTMn described above. This branch was not always as long as a normal bend but was most times of an appropriate thickness. In two animals, the contralateral giant fibers extended no presynaptic growth beyond the PSI synapse in a “bendless” phenotype (Figure 2.4E, red axon). As seen with the ipsilateral giant fiber, the contralateral giant fiber terminals were seen to dye couple to the TTMn in cases where the GFP had been adequately photobleached during imaging. Again, quantitation of this was limited due to the short window of time the prep remained viable for dual fills. Overall, with the methods available, the contralateral giant fiber appeared to be developmentally healthy based on growth through the neck, size of terminals, and presence of gap junctions. The growth of the contralateral giant fiber across the midline appeared directed toward the intact TTMn, as it followed the dendrite morphologically with no aberrant branches in any other directions. It is likely that these gross morphological changes were the direct result of the absence of its native synaptic partner.

2.3.4 Ablation of both postsynaptic motorneurons

To determine what role the dendrites as a whole play in the development of the giant fiber presynaptic terminals, I expanded the ablations to target both TTMn

cell bodies in the larval CNS. This experiment echoed previous work that was genetically able to prevent extension of both medial dendrites of the TTMn through the expression of a constitutively active form of the small GTPase Dcdc42(v12) (Allen et. al., 2000). Those experiments, however, did not remove the entire postsynaptic cell and relied on exogenous expression of proteins known to play a role in dendrite outgrowth. By ablating the motorneurons, I removed any possible effect they may play both through contact-mediated and long-range signals. This approach follows the logic of experiments from other systems with broad removal of target regions.

The ablation of both motor neurons was, as expected, less efficient than that of one. Electrophysiology was used to screen for the presence or absence of input to the TTMs. Animals in which one or both TTMs responded to stimulation were scored as non-bilaterally ablated. Only animals in which neither TTMn responded were considered positive bilateral ablations. Under those criteria, I achieved a bilateral ablation success rate of 8.9 % ± 6.9%. However, taking into consideration the 25% success rate for one TTMn, one would predict a success rate for both of around 6.25%, near the achieved rate. Connectivity of the giant fibers to the DLMs was shown to be intact and of normal latency (Figure 2.2). Survival rates for these experiments ranged from 40-80%. Locomotion effects of ablated adults were similar to what was observed in unilateral ablations trials.

2.3.5 Response of giant fibers to bilateral TTMn ablation

After confirming the absence of both TTMn cell bodies by GFP expression, both giant fibers were injected with Lucifer Yellow for anatomical characterization. It was less important to distinguish one axon over the other with separate dyes due to the symmetry of the ablation. All giant fibers descended through the neck connective with normal thickness and formed synaptic connections at the PSI. These giant fibers then failed to elaborate beyond that point, with no stereotypic bend in either direction (Figures 2.2; 2.4F). This result was similar to what was observed in the previous experiments using genetically stunted neurite outgrowth (Allen et. al., 2000).

2.4 Discussion

By eliminating the normal synaptic partner for an axon, I revealed an innate ability for pre- and postsynaptic elements to compensate. This uncovered a mechanism for plasticity, possibly used to ensure maximum connectivity in the escape response. This compensation was achieved through some interesting adaptations. The target-deficient giant fiber was able to defy midline repulsion, which functions in the wild type fly to restrict growth paths of bilaterally symmetrical homologs. Additionally, a circuit that normally exhibits one axon to one dendrite connectivity switched to a configuration in which two axonal terminals co-occupy a single target territory. These data together suggest a cell-autonomous response to

the absence of a postsynaptic signal leading to a switch from competitive "one axon-to-one dendrite" synaptic scheme to a cooperative synaptic scheme (multiple axons per dendrite). This appeared to be dependent on the axon's new ability to cross the normally restrictive midline. Still unclear is the degree to which the axon's ability to alter its guidance profile is connected to its shift in connectivity. It appears that there is some force preventing redundant synapses in the wild animal, though this restriction was lifted in the crossed orphan.

2.4.1 The TTMn supplies a factor required for giant fiber synaptic growth

The bilateral ablation experiments provided a chance to dissect possible interplay between presynaptic and postsynaptic elements. A classic approach to learn the function, if any, of a component within a whole is to remove it and observe the effect. By removing both postsynaptic cells, I eliminated the receptive dendritic field well before the nascent connections could form. This allowed me to assess the behavior of the giant fiber axons with no endogenous input from a native synaptic partner. If this input existed and played some role in proper development, subtracting that role should yield a visible effect. Previous work had suggested that the medial dendrites of TTMn play a crucial part in the growth of the giant fiber presynaptic terminals (Allen et. al., 2000). The Gal4-driven postsynaptic expression of a constitutively active form of the GTPase, CDC42(V12), was able to arrest neurite outgrowth from the TTMn cell body, causing it to not extend its medial dendrite. In these experiments, the absence of dendrites was correlated with the

absence of the stereotypic presynaptic “bend” by the giant fibers beyond their connections to PSI. This was the initial evidence that the formation of the giant fiber/TTMn presynaptic terminal is dependent on some factor provided by the postsynaptic cell. However, to confirm that it was the loss of a postsynaptic cue and not a side effect of the over-expression, we needed to completely remove the cell through non-genetic means, which was accomplished with laser ablation. As expected, our results confirmed these earlier findings. The giant fiber did not elaborate a presynaptic terminal posterior to the PSI connection in the absence of the TTMns’ medial dendrites. This strongly supports that presynaptic growth requires a postsynaptic factor. Further support comes from work presented by Godenschwege et. al. (2002b). This work demonstrated, through a manipulation of the midline push/pull signal, Slit/Roundabout, that when the postsynaptic dendrites were deflected laterally from the midline beyond some point, the giant fiber axons were no longer responsive to them, again failing to form a presynaptic terminal. This could be rescued by a complimentary deflection of the giant fiber axons laterally to meet the dendrites, which restored growth and connectivity. This suggested a distance dependent effect of the TTMn on giant fiber terminal formation, implying this signal was locally secreted, or even membrane bound. Based on the synapse between the giant fiber and PSI, which appeared normal by electrophysiological characterization, we can also infer that this hypothesized TTMn factor was neither necessary for the formation of the GF:PSI synapse, nor did it have an effect on the PSI development itself.

2.4.2 Asymmetric ablation causes cell autonomous response in deprived axon.

The removal of a single TTMn had a pronounced effect on the deprived axon, which showed reactive growth across the midline in most cases. This growth was along the dendrites of the intact TTMn, which were also occupied by ipsilateral giant fiber. While the non-deprived axon appeared normal in its growth terminal, the reaction of a deprived giant fiber sheds light on its dynamics in the following four distinct stages: recognition of an absent target, crossing of the midline, recognition of a non-native target, formation of a redundant presynaptic terminal.

The first observation is that the target-deprived giant fiber was able to actively respond to the absence of its endogenous target. The “response” itself will be examined below, but what I mean to emphasize here is that there is a measurable reaction by a cell cued by the lack of a signaling partner. It has already been hypothesized that presynaptic growth requires some factor from the postsynaptic cell. In the presence of this factor, it would seem that the giant fiber extends a presynaptic terminal, as seen in the wild type and non-deprived giant fiber. In the absence of this signal, the giant fiber didn’t simply “stall” at the PSI synapse, continuing to wait for a “GO” signal that would never come. Instead it responded by initiating a second mechanism by which it was able to recognize and grow toward other viable targets across the midline, perhaps with the same machinery used in its normal target recognition. It is worth noting at this point that in the case of a bilaterally ablated animal, this response was moot as there were no targets present,

native or novel. Also important to consider is the possibility that the reactive growth mechanism may share components with the normal synaptic pathway. Axonal growth cones are known to process several different inputs and weigh the balance, so it is conceivable that the observed response is mediated by a computational shift in the cone rather than a change in postsynaptic cue.

A second "stage" of the observed response came in the crossing of the midline, despite the presence of Slit/Robo repulsion (Godenschwege et. al., 2002b). As previously described, the giant fibers use this repulsive signal to maintain proper distance from the midline and never cross the midline in normal development, much like scores of other cells with bilateral symmetry across phylogeny. However, in this case, the target-deprived axon was able to grow toward and across the midline in over 90% of specimens. Clearly this barrier was non-active for the orphaned giant fiber. Looking at the ipsilateral axon response, we can assume the midline was still producing Slit, however. If a global reduction of Slit had occurred, the unaffected giant fiber would have had no barrier to keep it from growing along the dendrites of its synaptic partner, which extend across the midline – a result never seen. This leads to the first of two very important questions about the orphaned axon's behavior. How does it cross the midline?

Since the giant fibers are known to use the Roundabout receptor in their midline avoidance, it stands to reason that the signaling of this receptor was rendered inactive in the contralateral giant fiber somehow. This could be accomplished in a few ways. The giant fiber could stop new Robo production, either

transcriptionally or translationally, allowing natural protein recycling to remove older receptors. This relies on protein turnover rates, which have not been measured for Roundabout and likely exceed the relatively short developmental window in which the giant fiber first contacts the TTMn. Another option is the active removal of Robo from the surface by another protein, such as Commissureless, whose endogenous role is to remove Robo from the surface of growth cones (Kidd, 2009; Kidd et. al., 1998). It is also possible that downstream factors in the Robo signaling pathway are inactivated. I examine this guidance pathway in the chapter three.

The "how" of crossing is followed by the "why". What signal, if any, draws the axons across the midline? Perhaps the answer lies in the next "stage" of its unique response. Once in its homolog's territory, the giant fiber was able to respond to the intact TTMn, forming the characteristic lateral bend similar to wild type presynaptic terminals. This exogenous synaptogenesis provided support to an earlier hypothesis. Previously, I confirmed the dependence that giant fibers have for these dendrites. The presence of the TTMn dendrites was necessary for extension of giant fiber presynaptic terminals. However, the contralateral terminal in the ablated animal introduced the possibility that these dendrites may be sufficient to induce growth. Again, this is presumably attributed to some factor, produced by the TTMn. This result would support a model where this factor, produced by both TTMn is able to act equally on either giant fiber to induce terminal growth. Presumably, this influence is usually stifled in wild type due to a combination of midline repulsion and presence of the local source. It was only through the removal of its native partner that we

allowed a giant fiber to react to potential targets normally ignored. What was clear, however, was that this reaction is targeted. A crossed giant fiber appeared to only be extending reactive growth along the homolog of its native partner. This tells us that the hypothesized factor is likely unique to the TTMn, but identical between homologs. This strengthens the Giant Fiber System as a model circuit for studying competition. Our original definition of competition places requirements on both presynaptic and postsynaptic components. In order for a system to be defined as using competitive modeling, there needed to be an inherent similarity between competitors and target cells. The ubiquity of TTMn signals fulfils the requirement for postsynaptic uniformity. Competition would be one way to overcome this uniformity.

Finally, we come to the most intriguing aspect of the giant fiber response to unilateral ablation. In the wild type animal, each giant fiber forms a synapse with one and only one TTMn, the one on its side of the midline. However, following the removal of one TTMn, both giant fiber axons were able to positively respond to the same postsynaptic target, a situation never seen in normal animals. This may shed some light on the guiding forces behind giant fiber development. In wild type, the giant fibers normally adopt a “one-to-one” ratio when forming connections to the TTMns. This was hypothesized to be the result of a competitive mechanism, in place to eliminate erroneous and redundant connections in the escape circuit. However, the ability of two axons to be forced onto the same postsynaptic neuron suggests a different arrangement. It is possible that the restriction of a giant fiber to its native side is entirely dependent on the presence of Slit signaling through

Roundabout to prevent midline crossing. The role of this, and another guidance pathway, is examined in the next chapter of this dissertation. I would like to make note here, however, that this seemingly cooperative arrangement of two axons on the same dendrite has not been characterized synaptically. Current techniques are unable to probe the sharing of synaptic workload, which remains speculative based on dye-coupling observations. Instead electrophysiology probed the final muscular output of the circuit, which appeared normal. There remains the possibility given just the information presented that the similarity in growth between the axons is not a failing of competitive mechanism, but that an unequal contribution to synaptic transmission may be the true litmus test of competition.

2.4.3 Responsive growth by the intact TTMn suggests dendro-dendrtitc competition

The reaction by the medial dendrite of the intact TTMn provided some insight into the means by which the receptive target field is established for the giant fibers. In the wild type circuit, the TTMns extend their medial dendrites to meet at the midline, with minimal overlap, though they do contact at the distal tips. Extension beyond the midline has been noted in a few specific genetic backgrounds, though in the vast majority of control lines studied, the medial dendrites met at the midline and grew no further (Baird et. al., 1993). However, when we examined the baseline frequency of dendritic midline crossing in the ShakB:GFP genetic background, we did observe a 50% rate of crossing (Figure 2.3F, N). These projections were thin with little to no branches. This is contrasted by the medial dendrites of the intact

TTMn of a unilaterally ablated animal. Those projections occur more frequently (94%), and show higher order branching. While it is not the focus of this work, the reactive growth in territory by a neuron following the loss of its homolog was reminiscent of other work that examined the formation of tiled receptive fields.

The reactive expansion of a dendritic field into that of a homolog has most widely been studied in the tiled sensory dendrites of Drosophila embryos (Grueber et. al., 2003; Gao et. al., 2000). There, the individual processes have been shown to recognize dendrites from their homolog and avoid overlap. It is reasonable to think this may be occurring in some fashion between the homologous TTMns. Further investigation should be done to explore the variety of current proteins already implicated in self-recognition and the tiling of homologous dendrites, including Flamingo and DSCAM's 1 &2. The discovery that TTMn dendrites may use a mutual repulsion system in the formation of their receptive field further supports the Giant Fiber System as an ideal model system to study neuronal competition in its various forms.

In conclusion, the use of laser ablation has proven to be a technically viable and reasonably efficient way of eliminating one or both jump motor neurons in the Giant Fiber System. Using this system, I was able to uncover plasticity in the development of the giant axons. This involved a proposed cell-autonomous regulation of guidance by the target-deprived axon. Also of note was the change from a seemingly competitive synaptic ordering to one of overlap and redundancy, though these two states must be further characterized. It is important to keep in

mind at this point that only the axon on the ablated side showed a response. This could indicate a hierarchy of pathways, with compensatory plasticity only being used upon the failing of traditional guidance. They key observation in this result is the existence of an underlying plasticity, which can be exploited in the examination of neuronal competition.

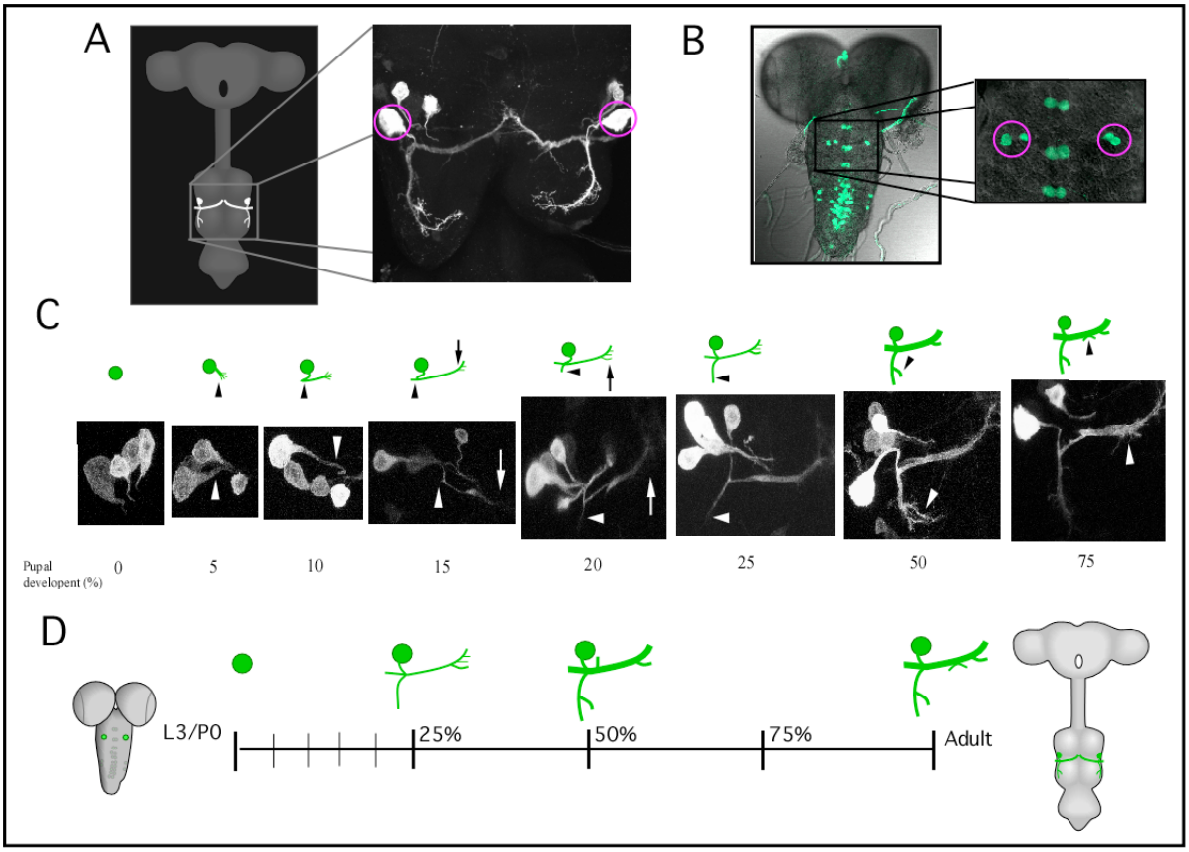


Figure 2.1. Development of the TTMn. Expression of GFP in the larval (A) and adult (B) CNS shows refinement of the driver pattern. (C) Staging of the TTMn lateral and medial dendrites using GFP expression (n=5 animals) through pupal development (100 hours at 25°C). At late larval/early pupal stags, the TTMn cell body is located in a cluster of four cells, all of the same size (approximately 20 mm) and expression intensity, making positive identification impossible. These cells extend primary neurites during the first 5% of pupation (arrowhead). While all extend toward the midline, the TTMn is the only to also divert ventrally. This process doubles back (C-10%, arrowhead) before extending to the midline (C-15%, arrow). The medial branch reaches the midline by 20% of development (arrow), but refinement of fine midline processes occurs over several hours, reaching the adult pattern midway through pupal development (C-50%). A lateral branch emerges from the deflection point in the medial process (C-15%, arrowhead) and continues to extend posteriorly (C-20%, 25%, arrowheads) and branches in the second thoracic neuromere (C-50%, arrowhead). (D) A cartooned representation of TTMn development from late larval to adult stages.

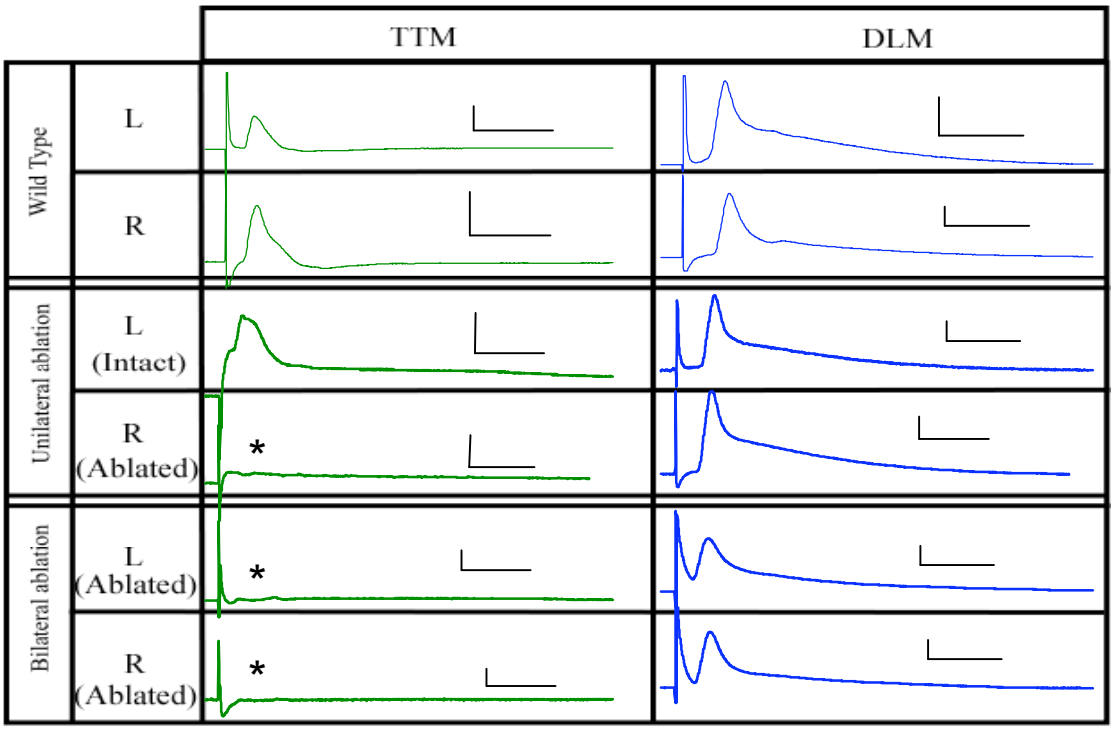
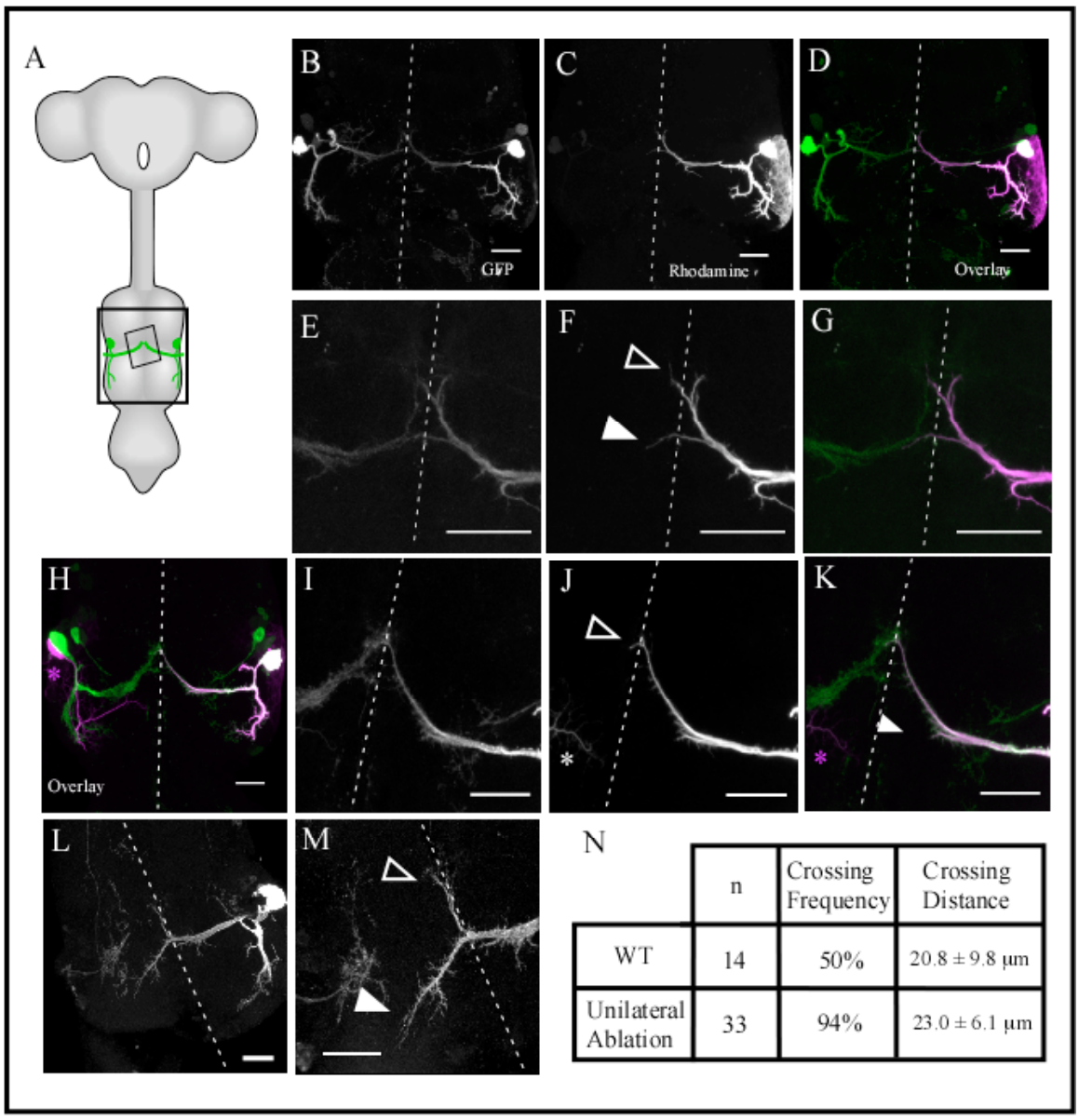


Figure 2.2 Electrophysiology of unilaterally and bilaterally ablated animals. The response latencies for wild type TTM and DLM are 0.8 and 1.4 ms respectively (first panel, ShakB-Gal4, UAS-GFP). Both muscles follow 100Hz stimulation with near perfect fidelity (not shown). In animals that have undergone ablation, the muscle corresponding to the ablated motorneuron (R-TTM) does not respond to GF stimulation, while the intact motorneurons (L-TTM, R-DLM, L-DLM) respond with wild type latency and following at 100Hz. Calibration bars are 20mV by 4ms



	n	Crossing Frequency	Crossing Distance
WT	14	50%	20.8 ± 9.8 μm
Unilateral Ablation	33	94%	23.0 ± 6.1 μm

Figure 2.3 Midline crossing of the TTMn medial dendrite. (A) The jump motorneurons, cartooned within the CNS, extend homologous dendrites to the midline. Dendrites of the TTMns were identified by ShakB-Gal4, UAS-GFP expression (B, E, I, L, M) and injection of rhodamine was used to image the dendrites of a single TTMn (C, F, J). Overlay confirms dye filling of GFP-positive dendrites (D, G, H, K). Higher magnification of the dendrites at the midline (E, F, G) shows minimal crossing of anterior (open arrowhead), and posterior (closed arrowhead) midline branches in half of animals tested (n=14). (H-K) In animals where crossing was not observed, the dendrite reaches the midline but did not branch. (L,M) Following removal of a TTMn, the intact motorneuron's medial dendrite crosses the midline in nearly every animal, closed arrowhead (94%, n=33). (N) Quantification of crossing frequency and average distance of extension across the midline. Distances given are only for those dendrites that crossed. Asterisks in H-K label an off-target rhodamine fill. Midline indicated by dashed line. Scale bars = 25mm.

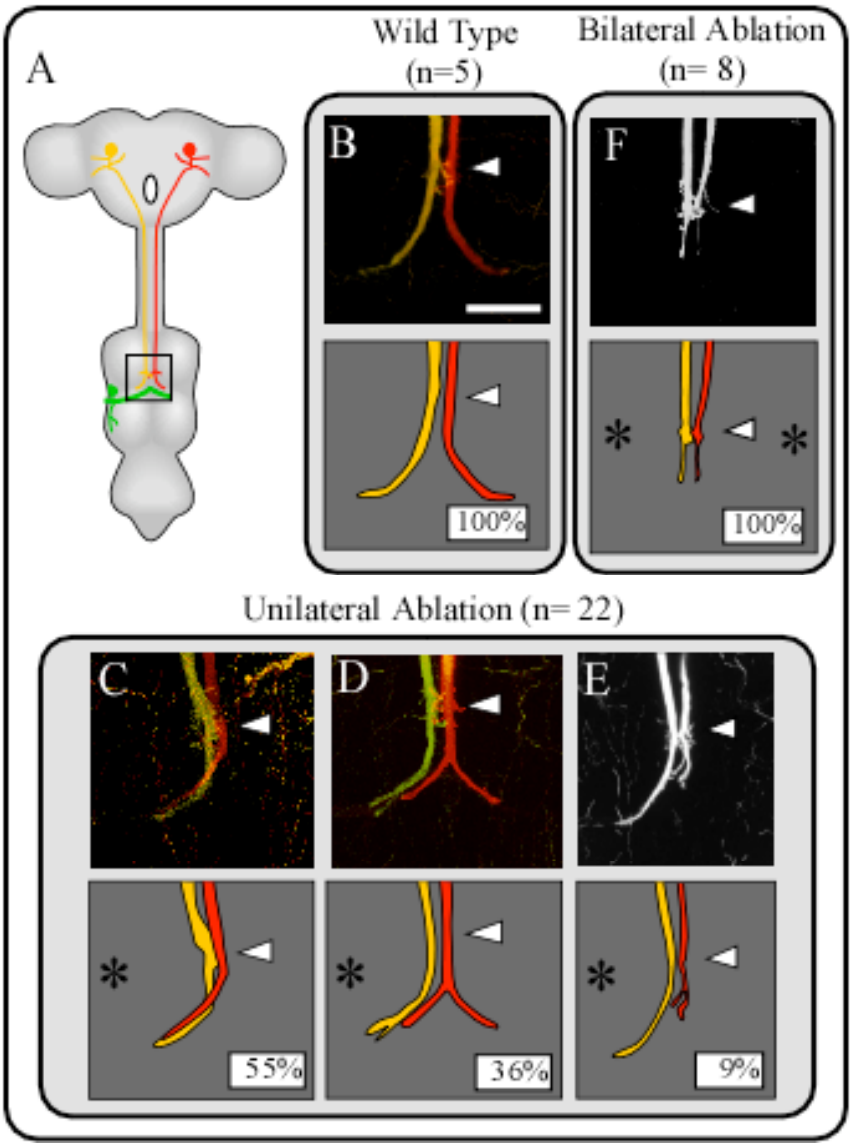


Figure 2.4. Target-deprived giant fibers respond with contralateral extension of presynaptic terminals. (A) The giant fibers, cartooned within the adult CNS, extend a presynaptic terminal to the TTMn posterior to GF-PSI synapse is indicated by arrowheads in B-F. (B) The presynaptic terminal forms a stereotypic “bend” is to the ipsilateral TTMn in wild type animals (ShakB-Gal4,UAS-GFP). (C-E) Unilateral ablation results in alteration in the orphaned giant fiber’s presynaptic terminal (asterisk denotes location of intact TTMn). (C,D) In most cases, the target-deprived GF crossed the midline and elaborated a terminal along the intact dendrite. In 55%, this terminal was solely contralateral (C), while 36% branched and grew bilaterally, growing along dendrite on both sides of the midline (D). In all animals, the non-deprived GF (yellow in C-E) formed a normal ipsilateral terminal to the intact TTMn. (E) In two cases, the orphaned GF extended no presynaptic terminal beyond its connection to the PSI. (F) Bilateral removal of both TTMns resulted in neither GF elaborating a terminal past the PSI. Scale bar = 25 mm

CHAPTER 3
EXAMINATION OF REPULSION AND ATTRACTION: GUIDANCE DYNAMICS IN THE GIANT FIBER SYSTEM

3.1 Introduction

The ablation experiments presented in the previous chapter probed the Giant Synapse of Drosophila for competitive interactions. Removal of a postsynaptic target caused the deprived axon to cross the neural midline and form its presynaptic terminal along the contralateral TTMn. The giant fiber crossed a repulsion barrier and exhibited directional growth toward the new target. This raised two questions regarding the axon's guidance, namely how was it able to cross the normally repulsive midline, and what, if anything, was promoting this directional growth? This midline crossing phenotype showed that the giant axons are plastic in their guidance; able to adapt to perturbation with dynamic alteration of their native guidance responses. To better understand this giant fiber reaction, I examined the roles of two canonical guidance pathways, one repulsive and one attractive, as they pertain to the Giant Fiber System.

3.1.1 Repulsion through the Slit/Roundabout Pathway

To address the target-deprived giant fiber's ability to cross a known midline barrier, I first examined the ligand/receptor pair of Slit/Roundabout. These two proteins have been characterized in nearly all neural model systems, including *Drosophila*, as having a repulsive role during axon guidance (Williams et. al., 2004;

Guan & Rao, 2003; Bashaw 2000; Brose et. al., 1999). The ligand, Slit, is a secreted protein produced typically by cells at key decision points or along tracts to direct growth cones (Williams et. al., 2004). Early work identified Slit as a chemotaxic molecule due to its ability to both induce outgrowth of axons from neural explants but also direct that growth away from a source of Slit (Brose et. al., 1999). This repulsive growth is the result of Slit being bound by proteins in the Roundabout (Robo) family, expressed on growth cones. Once bound, the pair signal through the Rac family GTPases to trigger growth away from the Slit source (Bashaw et. al., 2000). In *Drosophila*, this role has been identified most notably during embryonic and larval neural tract formation, where axons are held on one side of the midline. Additionally, the expression of Slit by midline glia is translated into the lateral position of longitudinal axon tracts (Zlatic et. al., 2009; Godenschwege 2002b). This is accomplished through the careful regulation of three members of the Roundabout family in embryonic stages (Guan & Rao, 2003; Kidd et. al.,1998). By expressing different combinations of Robo1, Robo2, and Robo 3, axons are able to establish different sensitivities to a single Slit point source. The more members of the Roundabout family an axon expresses, the more sensitive it is to Slit. This causes it to grow at a constant distance from the midline, determined solely by the Slit concentration gradient (Fan et. al., 2003).

However, some of these Slit-sensitive axons do need to cross the midline, usually once, and at very specific places. This is tightly regulated through the expression of a protein named Commissureless (Comm), whose function is the

down-regulation of Robo1 protein (Kidd, 2009; Tear et. al., 1996). This is primarily thought to occur indirectly, with Comm binding newly produced Robo within the cell, shunting it off to lysosomes; thereby preventing it from reaching the cell surface (Keleman et. al., 2005; Couch & Condron, 2002; Rosenzweig & Garrity, 2002). However, contrary papers have provided evidence suggesting that Commissureless may also actively remove mature Roundabout receptors from the surface of neurons. In the proposed mechanism, Comm is directed to the surface where it binds to Robo. The complex is then marked for internalization and destruction through ubiquitination by DNedd4, a ubiquitin ligase (Ing et. al., 2007; Kanelis et. al., 2006; Georgiou & Tear, 2003; Myat et. al., 2002). Still some models propose a unified and redundant co-existence of both silencing pathways (Gilestro, 2008). This multi-path removal of Robo serves to allow growing axons to cross the midline at very specific locations along the developing nerve chord. Once across, the cell turns off *commissureless* expression, allowing Roundabout to return to the surface. The receptor binds Slit, and signals repulsion, preventing re-crossing of the midline. This temporal regulation of commissureless thus translates to a very specific spatial patterning of midline crossing.

The Slit/Robo system has also been shown to be vital to the proper wiring of adult circuits (Godenschwege et. al., 2002b; Wolf et. al., 1998; Chiba et. al., 1993). In the case of the giant fiber circuit, expression of *roundabout1* restricts the axon from crossing the Slit-rich midline (Godenschwege et. al., 2002b). It stands to reason that any situation in which a giant fiber is able to cross this barrier must be, in

part, due to a change in Robo1 signaling within that cell, whether by direct or indirect mechanisms. This raised the question of whether reduction of Robo is sufficient to allow for midline crossing. In the first part of this chapter, we examine the giant fiber response to changes in functional Roundabout levels.

3.1.2 Attraction through the Netrin/Frazzled Pathway

Following ablation of its native synaptic partner, the giant fiber grew directly across the midline where it elaborated its lateral bend along the dendrites of the TTMn on the opposite hemisegment. As shown in the previous chapter of this dissertation, the extension of the giant fiber's stereotypic synaptic terminal with the TTMn was entirely dependent on the presence of the TTMn dendrites. When they are removed, giant fibers did not elaborate their characteristic "bend" beyond the PSI connection. Together, these observations suggested the presence of some attractive cue, locally unique to the TTMn and identifiable by the giant fibers. The known attractive role of Netrin in neural development and guidance decision-making, combined with the suggested presence of an unknown attractant in the giant fiber system, placed the Netrin/Frazzled pair as prime candidates.

Additionally, if giant fibers utilize a ligand-based mechanism to ensure non-redundant target selection, it may benefit the system to use a single ligand that has receptors of opposing function. In this case, Netrin's receptors, Frazzled and Unc5, offer a possible "push" and "pull" signals, potentially guiding giant fibers away from one TTMn and towards another. The Netrin family of proteins (Unc6 in *C. elegans*)

is considered one of the canonical guidance signals. These secreted ligands act largely through the Deleted in Colorectal Cancer (DCC) family of receptors to signal axonal outgrowth and chemoattraction. This receptor family, including DCC and neogenin in vertebrates (Keino-Masu et. al., 1997, 1996), Unc40 in *C. elegans* (Chan et. al., 1996), and Frazzled (Fra) in *Drosophila* (Kolodjiej, 1997; Kolodziej et. al., 1996), has a conserved extracellular structure consisting of six immunoglobulin and four fibronectin domains as well as three conserved intracellular domains, P1, P2, and P3 (Garbe & Bashaw, 2007). Similarly, the P2 domain of DCC/Frazzled has been identified as the domain responsible for its interactions with the Roundabout receptor (Stein & Tessier-Lavigne, 2001). It is believed that DCC/Unc40/Fra functions through multimerization, facilitated at the P3 domain, though the exact configuration and number has yet to be examined (Stein & Tessier-Lavigne, 2001). Downstream signaling of DCC/Unc40/Fra is still yet to be entirely established, but it has been suggested that all three identified intracellular domains recruit additional factors, such focal adhesion kinase (Fak), Unc34/Enabled, and Ced-10, a Rac GTPase (Garbe & Bashaw, 2007). It has also been suggested that additional, unidentified partners exist for this receptor.

In addition to the complex, attractive signal Netrin induces through the DCC family, there is a second pathway acting through the Unc5 receptor to produce a repulsive signal (Brankatschk & Dickson, 2006). This pathway has been characterized in spinal axon cultures and *C. elegans* (Keleman & Dickson, 2001). Interestingly, not only can Unc5 direct signal repulsion over long range, but the co-

expression with DCC can also shift that receptor's attraction to a short-range repulsion (Keleman & Dickson, 2001).

The ability for Netrin to trigger both attractive and repulsive growth make it an intriguing candidate for study in light of the deprived axon's reactive growth. The axon's ability to switch from being directed away from the midline to drawn across toward a surrogate target led us to investigate the roles Netrin and Frazzled play in giant fiber development. These studies provided an intriguing compliment to the Slit/Robo experiments.

3.2 Methods

Drosophila Stocks. All stocks were raised on standard medium at 25°C unless otherwise noted. The following stocks were used: (1) $comm^5$ (Tear et. al., 1996), (2) CyO/sp; UAS-commissureless (3) UAS-comm; CyO/sp (4) shaking-B/FM6 (Jacobs et. al., 2000), (5) ShB/FM6; UAS-comm. (6) UAS-netrinB/Tm6 (7) UAS-netrinA/CyO (8) UAS-frazzled/CyO (9) UAS-frazzledΔC/CyO (10) UAS-Fra:Robo/Tm6 (11) UAS-dicer/CyO (12) CyO/sp; UAS-antiRobo1(RNAi)/Tm6 (13) CyO/sp; UAS-antiFrazzled (RNAi)/Tm6 (14) UAS-unc5/Tm6. There were four p[GAL]4 drivers used in this section to express UAS constructs within the giant fibers. The A307 driver has strong expression in the presynaptic giant fibers as well lower expression in the postsynaptic dendrites of TTMn (Allen et. al., 1998). The c17 driver is limited to only presynaptic expression (Godenschwege et. al., 2002b), while the ShakB-Gal4 driver

is exclusively postsynaptic (Jacobs et. al., 2000). The driver c42.2 expresses only presynaptically as well, but expression begins at around 50% of pupal development, well after the giant fibers have connected to the TTMns (Godenschwege et. al., 2002).

The single deletion mutants (NetAΔ and NetBΔ) obtained from the Dickson lab are characterized in their 2006 paper. Briefly, imprecise excisions of two P-elements (BG02298 and NP6098), were used to generate deletions in the promoter regions and coding sequence for Netrin A and B, respectively. Removal of the former resulted in deletion of 367 codons from netrin A (4816bp); the latter deleted 370 codons of netrin B (4184bp). The double loss of function (NetAΔBΔ here, NetABΔ in referenced paper) was accomplished through precise excision of two cis P-elements flanking the entire netrin coding region, NP6098 and KG04428. This excision removed 147kb, covering both netrin genes (Brankatschk & Dickson, 2006).

The NetAΔB^{myc} and NetAΔB^{tm} lines were generated using the NetAΔ line as a background. Homologous recombination was used to modify the 3' end of the endogenous coding region of netrin B in one of two ways. The first added a c-myc tag, transmembrane region, and intracellular V5 epitope tags (NetAΔB^{tm}). The second added only the c-myc tag (NetAΔ^{myc}). In both lines, the modified NetB replaced the endogenous and, given the deletion background, was the only Netrin present in the animal. While the version containing a transmembrane region remained tethered to the source cells, the c-myc labeled version was freely secreted

and is referred to in this dissertation as NetAΔB^{sec} to distinguish it from the transmembrane form, since both are c-myc labeled.

Temporal expression of UAS-Comm using the TARGET system. We applied the TARGET system to our examination of UAS-*commissureless* in the giant fibers. By recombining the A307-Gal4 and tub-GAL80^{ts} chromosomes, we were able to temporally express in the giant fibers. This was accomplished by crossing flies with the above recombinant second chromosome with flies carrying UAS-*commissureless* on the third chromosome (A307-p[GAL]4:tub-GAL80^{ts}/CyO; TM6β/MKRS x CyO/sp; UAS-comm/UAS-comm). The progeny were crossed to establish a balanced line (A307-p[GAL]4:tub-GAL80^{ts}/CyO; UAS-comm/TM6β). This line was maintained at the permissive temperature for GAL80^{ts} (22°C was sufficient to see no effect from UAS-*commissureless*). Developing flies were heat-pulsed by 24 hour rearing at 30°C. In brief, P0 pupae were collected and placed at 30°C for 24 hours, equating to roughly 30% of pupal development. After the 24 hours, they were placed back at 22°C until eclosion. Adults (1-3 days post eclosion) were examined anatomically for errors in the elaboration of the giant fiber presynaptic terminal.

Electrophysiology. Adult flies were examined electrophysiologically according to methods described in Chapter Two of this dissertation.

Presynaptic Dye Injections. Visualization of giant fiber morphology was done following methods described in Chapter Two of this dissertation. Analysis and post-processing, including Extended Depth Of Focus, volume rendering, and 3D Blind Deconvolution was performed as needed in NIS-Elements Advanced Research (Nikon Instruments, Melville NY).

Confocal Microscopy. Central nervous systems were dye-injected and prepared following methods described in Chapter Two of this dissertation. Fixed and cleared samples were mounted and placed under a 40x oil immersion objective (Nikon Plan Fluor 1.3NA, W.D. = 0.2mm) unless otherwise indicated. Confocal Z series were collected using an upright Nikon 90i microscope and C1si scan head. Collected spectral image stacks were linearly unmixed to resolve individual fluorophores using individually collected reference spectra.

3.3 Results

3.3.1 Reduction of Roundabout signaling allows midline crossing

To reduce the functional levels of Roundabout, the A307-Gal4 driver was used to overexpress UAS-*commissureless*. Giant fiber anatomy at the second thoracic neuromere was screened for changes in presynaptic terminal morphology (Figure 3.1A, n=131 animals). As the focus was initially on the morphological response of the giant fiber (i.e. midline crossing), anatomical rather than

electrophysiological characterization was used in these animals. Three distinct phenotypes were observed. In over 50% of the animals tested, the giant fibers were anatomically normal, with each forming the stereotypic lateral bend toward the ipsilateral TTMn (Figure 3.1B). In 31% of animals, this pattern was reversed, with each axon crossing the midline and elaborating their bend entirely toward the contralateral TTMn (Figure 3.1C). The remaining animals had at least one giant fiber split to form a bilateral presynaptic terminal (Figure 3.1D). The bilateral giant fiber was paired with giant fibers that either remained ipsilateral, grew contralaterally, or also split bilaterally (Table 3.1). Overall, if we count individual giant fibers, rather than examining pairs per animal, 53% of terminals extended ipsilaterally, 34% extended contralaterally, and 13% split bilaterally (n=262 giant fibers). All giant fiber lateral bends, regardless of side, were formed posterior to the connection to the PSI, in the region of the second thoracic neuropil occupied by the wild giant synapse. Dye coupling to the TTMn was observed in a number of cases, though frequency was not quantitated across the genotype (Figure 3.1). These terminals were anatomically identical to wild terminals in length and thickness with no aberrant processes or branching (beyond bilateral symmetry). This made anatomical characterization clear and categorical.

There was significantly less penetrance of these phenotypes when using other Gal4 drivers (Table 3.1). The presynaptic driver c17-Gal4 localizes expression solely to the giant fibers, but at much weaker levels. Unilateral expression has even been noted in some preps where c17 drove UAS:lacZ. Of the animals tested, 83%

connected ipsilaterally like wild type (Table 3.1, n=62). This was only decreased to 71% by doubling the copies of c17-Gal4. Postsynaptic expression with the ShakB-Gal4 driver produced no change from wild type (Table 3.1, n=28). Based on the inability of UAS-*commissureless* to affect either giant fiber when driven by the strong ShakB-Gal4, it is hypothesized that it is the presynaptic component of the A307-Gal4 driver expression pattern that yields the observed phenotypes.

Timing studies were performed to determine if UAS-*commissureless* expression was most effective at specific times during development. The C42.2-Gal4 driver expresses strongly in the giant fibers, but turns on at approximately 50% of pupal development, well after the giant fiber circuit is formed. Animals with UAS-*commissureless* driven with this C42.2-Gal4 were observed to have no crossing or novel connectivity phenotype (Table 3.1, n=15). However, utilizing a temperature sensitive form of Gal80, I was able to turn on expression of A307-Gal4; UAS-*commissureless* during the first quarter of pupation, when the circuit is forming. In these animals, giant fibers exhibited similar anatomical phenotypes to the A307-Gal4 study in which expression was constant through pupation. This study was limited in scope, but effective as a proof of concept.

The A307-Gal4 driver was used to express an anti-Robo RNAi UAS construct. Using this construct in the absence of UAS-*dicer* co-expression yielded no change from wild type anatomy. However, even with the co-expression of UAS-Dicer, low penetrance of “Commissureless”-like phenotypes was observed (Table 3.1). Though 74% of giant fibers remained ipsilateral, crossing and bilateral axons were observed

(n=19). This lower penetrance of the crossing phenotype could be due to the division of expressed Gal4 between two UAS constructs.

3.3.2 Increases in Frazzled permit midline crossing; Netrin elicits no change.

To examine a possible role for the Netrin pathway in the giant fiber system, I first tested alterations in the level of Netrin, using both over-expression and various loss of function mutants (Table 3.2). By over-expressing the attractant both pre- and post-synaptically, I aimed to first explore whether simple over-expression of this canonical attractant would affect the giant fibers. However, over-expression of either UAS-*netrinA* or UAS-*netrinB* produced no observable effect on giant fiber development. This was true whether expression was driven presynaptically with c17-Gal4 (n=10 animals for NetA; 8 for NetB), postsynaptically with ShakB-Gal4 (n=14 for NetA; 8 for NetB), or a combination of both with A307-Gal4 (n=12 for NetA; 12 for NetB). However, it was possible that over-expressing Netrin using Gal4 promoters specific to the Giant Fiber System may overlook any impact that sources outside the circuit may have on giant fiber development. To address this, I decided to affect global Netrin levels.

To examine a wider role for Netrin in the Giant Fiber System, I then turned to loss of function mutants. However, due to the critical role Netrin signaling plays during early development, I was unable to raise adult animals with homozygous mutations in both Netrin A and Netrin B. Instead, I examined various combinations of deletion mutations to gauge the effect of a reduced netrin expression profile.

Animals homozygous for the Netrin A deletion mutation (NetAΔ) developed normally with no discernable effect on the giant fibers (n=12 animals). The same was observed for NetrinAΔBΔ heterozygotes (n=8 animals). Animals with a homozygotes Netrin B loss of function (NetBΔ), however, showed terminal fraying in a third of animals tested (n=9 animals). These frayed giant fibers were normal in their ipsilateral bend posterior to the PSI synapse, but formed a bifurcated terminal with both branches contacting the ipsilateral TTMn. Outside of this local branching, giant fibers appeared normal in their overall morphology. No midline crossing or extension outside the wild type synaptic region was observed. Axons terminals appeared normal in length and thickness.

Along with all the discussed Netrin deletion mutants, two unique disruption/replacement constructs were also obtained from the Dickson lab to allow for tracking and localization of Netrin (Brankatschk & Dickson, 2006). Both expressed a modified form of the endogenous Netrin B in a NetAΔBΔ background. The first added a simple c-myc tag while the other tethered the produced Netrin by adding a c-myc tag as well as a transmembrane coding section and intracellular epitopes. In these animals, the modified form of Netrin is the only form expressed, given the deletion background. Ipsilateral fraying, similar to that seen in NetBΔ homozygotes was observed in animals in which the tethered Netrin was expressed (n=7 animals). However, with this tethered Netrin, additional extension of the giant fiber beyond the second thoracic neuromere was also observed. This growth appeared untargeted, traveling along the midline into the third thoracic or even

abdominal neuromeres. It is worth noting that these extensions were seen in addition to ipsilateral bends that appeared normal in their contact with the TTMn, perhaps indicating an error not in target identification, but rather in a "stop/go" signal.

Next, I examined the role of Netrin's attractive receptor, Frazzled (DCC) in the giant fiber system. Over-expression of a single UAS-*frazzled* transgene with the A307-Gal4 driver (n= 24 animals) disrupted giant fiber synaptogenesis in nearly 30% of animals tested, preventing giant fibers from extending a synaptic terminal in a "bendless"-like phenotype (Figure 3.2C-F). While the majority of these stunted giant fibers were paired with a homolog that remained ipsilateral (Figure 3.2C), some homologs crossed contralaterally (Figure 3.2D), split bilaterally (Figure 3.2E), or were bendless themselves (Figure 3.2E; Table 3.2). As expected, giant fibers that did not form a lateral bend were electrophysiologically affected as well, having no or large delays in response to direct brain stimulation and an inability to follow train stimulation (Figure 3.3E). Giant fibers that formed exclusive connections to one TTMn formed synapses with wild type latencies and normal train following (Figure 3.4A-C). Interestingly, in animals where both TTMns were innervated exclusively by a single, bilateral giant fiber, the response latencies were increased and showed a decreased ability to follow train stimulation at 100Hz and 200Hz (Figure 3.2E; Figure 3.3D), suggesting an inverse correlation between the size of a synaptic terminal and its efficiency.

Increasing the dosage of UAS-*frazzled* to two copies resulted in an interesting shift in morphological phenotypes (n= 13 animals). While the occurrences of

bendless giant fibers dropped from 29% to 8%, the frequencies of crossed and bilateral giant fiber terminals both increased (4% to 19% and 10% to 38% respectively, Table 3.2). This shift from “no terminal” to “non-wild terminal” meant an overall drop in ipsilateral connections. The percentage of individual giant fibers forming wild type bends dropped from 56% to 35%. This change is best described as a switch from a poorly developed, side-restricted system to one in which fibers cross freely and form overlapping terminals. In fact, in animals with a double dose of UAS-*frazzled*, 31% of all TTMns were contacted by both giant fibers, as opposed to 2% in animals with a single dose. Interestingly, driving expression with neither the weak presynaptic c17-Gal4 nor the postsynaptic ShakB-Gal4 led to any change in giant fiber morphology.

Silencing experiments were also conducted to examine the effect reduction in Frazzled would have on the giant fiber system. Expression of an UAS:RNAi construct against *frazzled* was driven with A307-Gal4. No appreciable change was observed in the giant fibers, even with a co-expression of a UAS-*dicer* construct. However, the high mortality rates of Frazzled null animals suggested this complete lack of phenotype was due more to effectiveness of the RNAi than a removal of Frazzled.

The ineffectiveness of RNAi to produce a phenotype led to the exploration of other constructs to reduce Frazzled function. One particular UAS construct, in which the cytoplasmic tail of Frazzled is removed (UAS-f*razzledΔC*), was shown to have a dominant negative effect on normal Frazzled function in embryonic studies (Garbe &

Bashaw, 2007). Expression of this construct driven with A307-Gal4 (n= 54 animals) resulted in the appearance of bilateral giant fibers, dual-contralateral fibers, as well as a phenotype common to the ablations. In some animals, one axon crossed the midline and shared the dendrite with the other axon, forming a unique case in which one TTMn hosted two giant fibers, while the other had neither (Table 3.2). While these non-wild phenotypes were seen with low penetrance (<25%), they demonstrated clear midline crossing and overlapped synapses similar to those seen in the ablated animals described in Chapter Two.

Interestingly, increasing the dose of the UAS-f*razzledΔC* construct resulted in nearly all the animals having disrupted electrophysiology and clear guidance errors. Many giant fibers exhibited high levels of branching, overextension, and untargeted growth through the CNS (not shown).

Netrin's second receptor, Unc5 was also examined for a role in the giant fiber system. Over-expression of UAS-*unc5* with the A307-Gal4 driver led to the complete functional and structural disconnection of the giant fiber from both PSI and TTMn. Additionally, no axon could be located in the neck connective for anatomical analysis through dye injection. The Unc5 receptor has a known repulsive function in response to Netrin binding. In light of this and the physiological and anatomical evidence, it is reasonable to assume the giant fibers were also unable to grow out from the brain in these animals. Further study into this receptor's role in giant fiber target recognition would require timed expression of the UAS construct in the narrow

developmental window between when the giant fibers leave the brain and when they reach the second thoracic neuromere.

The *unc5* over-expression phenotype was supported by experiments in animals expressing chimeric Frazzled/Roundabout receptors. These receptors were composed of the extracellular domains of Frazzled paired with internal Roundabout signaling domains. As characterized in embryonic studies, these chimeras respond to Netrin in a Frazzled-like manner, but elicit an intracellular Roundabout-like response (Stein & Tessier-Lavigne, 2001; Bashaw et. al., 2000; Tessier-Lavigne & Goodman, 1996). Specifically in the tested adults, giant fibers did not extend their axons down the neck connective, but showed guidance defects within the brain. This is presumably due to Netrin at the midline of the brain, which would cause a Roundabout-mediated repulsion and prevented axons from leaving the brain (unpublished Murphey lab data).

3.3.3 Reduced repulsion permits enhanced attractant phenotype

The appearance of overlapping terminals in animals over-expressing UAS-*frazzled* raised questions about the role of attraction in giant fiber competition. Interestingly, the increased rate of giant fibers co-occupying a TTMn was complimented by a rise in midline crossing. However, overexpression of Netrin did not result in a single giant fiber crossing the midline barrier, preventing me from examining its role in competition. To assess the effects of Netrin on a giant fiber that was capable of crossing the midline, animals were generated in which A307:Gal4

drove both UAS-*commissureless* and UAS-*netB*. The overexpression of *commissureless* had been shown to permit axonal crossing at the midline, presumably through its endogenous function of eliminating Roundabout signaling. I chose to focus on Netrin B specifically over Netrin A due to the mild phenotype in the *netrinB* loss of function mutants the availability Netrin B expression mutants for any later studies.

Initial, electrophysiological testing demonstrated longer latency, with the average TTM response extended to 1.44±0.66 ms (n=22 animals, direct brain stimulation). Response of the DLMs was unaffected with responses at 1.19±0.15 ms (Figure 3.4C, D). Correlation of specific electrophysiological defects to anatomical morphology will be discussed following description of the morphological effects.

Anatomical characterization through dye injection revealed a substantial increase in the number of giant fiber terminals that grew bilaterally and contacted both TTMns. Of the forty-eight giant fibers examined (24 animals), three-quarters of them were bilateral (Figure 3.4B, C). Bilateral giant fibers formed two symmetrical terminals, one to the ipsilateral TTMn and the other to the contralateral TTMn. These terminals were generally of an appropriate length and width compared to wild type. Some terminals were slightly longer and thinner but still in close contact with the medial TTMn dendrite. Phenotypically, the most common result was an animal with two bilateral giant fibers (58%, Figure 3.4B, C), followed by a bilateral paired with an ipsilateral giant fiber (29%). Pairing of a bilateral with a contralateral giant fiber, as well as dual contralateral and dual ipsilateral giant fibers were represented

in a single case each. These distributions translated to 75% of TTMn dendrites hosting terminals from both giant fibers. However, there appeared to be a correlation, as before, between the expansion of a terminal bilaterally and the function of synapses created. In cases where both axons formed bilateral terminals, the TTMns had diminished function. The response latency at the TTM in these animals was 1.48±0.61 ms. Similarly, in animals where a bilateral was paired with a unilateral giant fiber (ipsilateral or contralateral), the side receiving dual input had a nearly wild type response, with latencies of 1.07±0.55 ms, which is just above the one millisecond cut off for a wild type categorization. However, the side receiving a single input, from a branch of the bilateral giant fiber had an average latency of 1.73±0.76 ms (Figure 3.4C). Overall, expansion of bilateral terminal was correlated to a reduction in its synaptic function to both TTMns.

3.4 Discussion

In the second chapter, I probed the Giant Fiber System for competition using ablation in the classic paradigm. The resulting crossed giant fibers raised questions about the nature of guidance in the giant fibers. Namely, could we replicate the crossing by altering levels of repulsive or attractive cues? Reduction of repulsion receptors allowed for axonal crossing, as did over-expression of attraction receptors. However, the results also carry implications for competition. Specifically in the case of *commissureless* overexpression, the crossed axons maintain a one-axon-per-

dendrite configuration, similar to wild type, rather than the 2:1 seen in ablated animals. Furthermore, if simply shifting the balance of repulsion and attraction causes can cause crossing, why was this phenotype not seen following Netrin overexpression? Was the midline repulsion too strong to overcome? These questions led to the combination of elevated Netrin in a background of elevated Commissureless. Interestingly, giant fibers not only cross freely, indicating a reduction in repulsion, but also form overlapped connections on both sides of the midline, indicating a marked shift in proposed competitive forces.

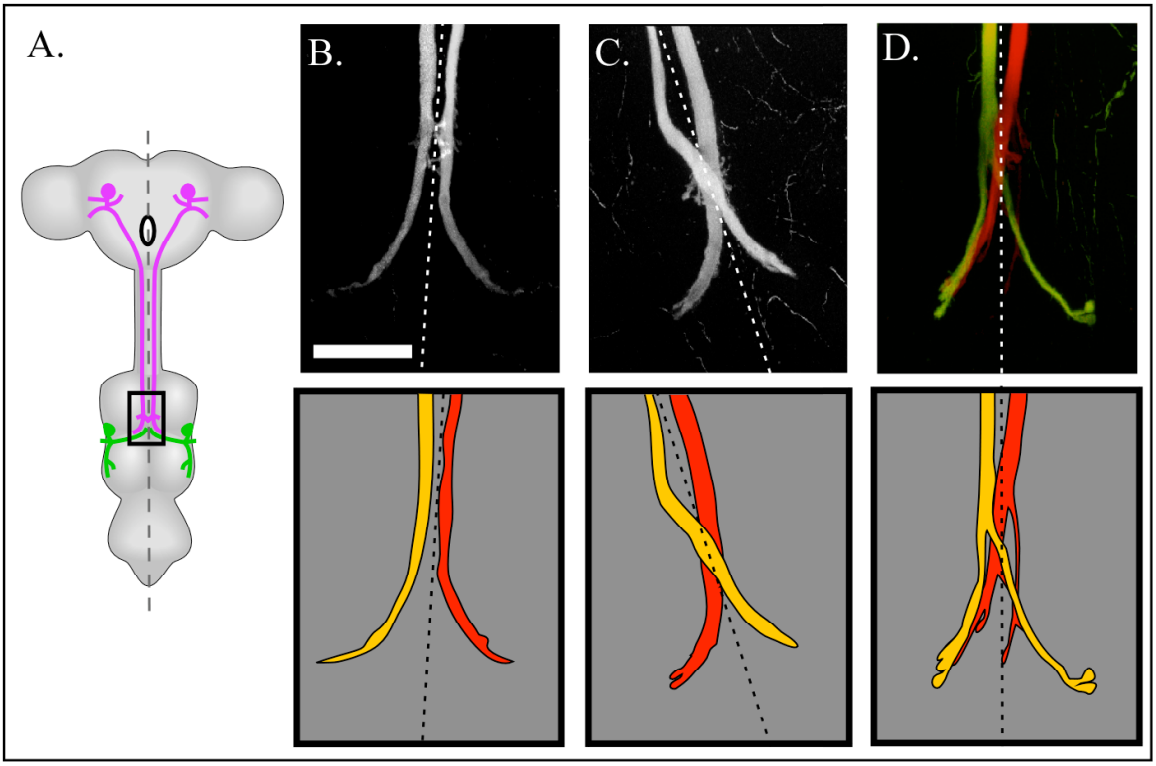


Figure 3.1. Overexpression of UAS-*commissureless* causes giant fiber midline crossing. (A) The giant fibers (magenta) connect to the TTMns (green) of the ipsilateral side in wild type animals. (B, C, D) The giant fibers of animals with UAS-Commissureless driven by the A307-Gal4 driver exhibit three distinct anatomical phenotypes. (B) Giant fibers maintained "wild type" anatomy in 50% of animals tested. (C) Over 30% of giant fiber pairs crossed the midline and synapsed with the contralateral TTMns. (D) In less than 20% of animals, at least one giant fiber formed bilateral terminals. n= 131 animals. Scale bar = 25um.

Table 3.1. Phenotypic distribution of direct and indirect Roundabout reduction. Frequencies of each phenotypic outcome are shown for each genotype tested in the Roundabout and Commissureless studies. Frequencies are given in percentages of the number of animals tested in that genotype (given under the "n" column). Dashes within the chart indicate no presence of that phenotype.

Genotype	n								
A307:GAL4	9	100	-	-	-	-	-	-	-
A307:GAL4; UAS-*comm*	131	5.4	-	31.3	6.1	5.3	6.9	-	-
c17:GAL4; UAS-*comm*	62	88.7	-	-	9.7	-	1.6	-	-
ShakB:GAL4; UAS-*comm*	28	100	-	-	-	-	-	-	-
C42.2:GAL4; UAS-comm	15	100	-	-	-	-	-	-	-
A307:Gal80ts; UAS-Comm P0-24hr	5	40	-	40	-	-	20	-	-
c17; UAS-Comm/UAS-Comm	14	71.4	-	-	21.4	-	-	7.1	-
A307:Dicer#3;UAS-antiRobo1	19	73.7	-	5.3	15.8	-	-	-	5.3

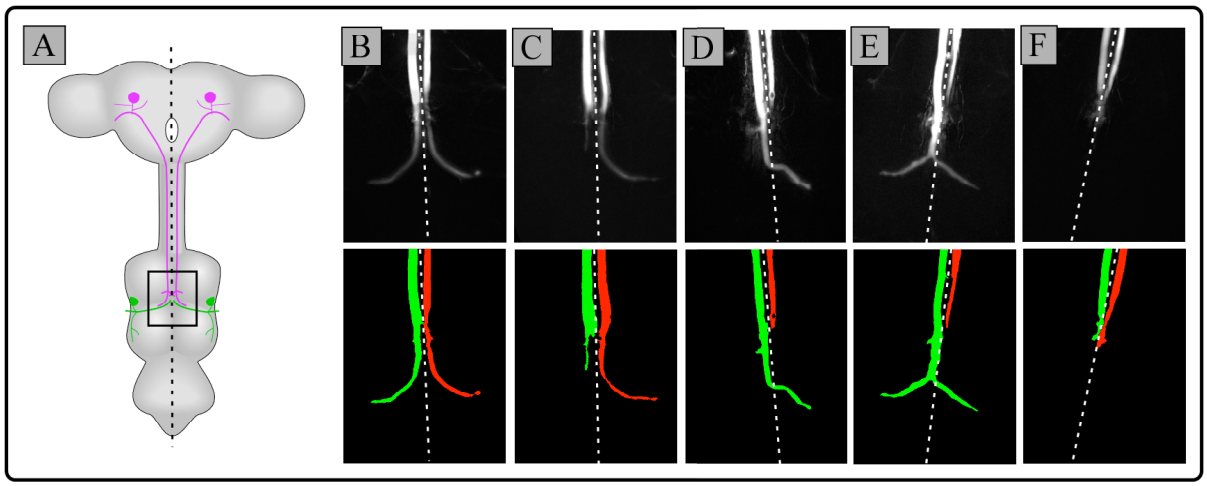
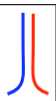
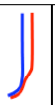
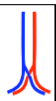
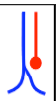
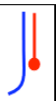
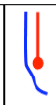
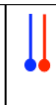
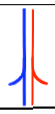
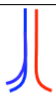
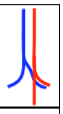
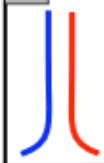


Figure 3.2. Overexpression of UAS-*frazzled* causes a loss of presynaptic terminals. A) The giant fibers (magenta) connect to the TTMns (green) of the ipsilateral side in wild type animals. B-F) The giant fibers of animals with UAS-*frazzled* driven by the A307-Gal4 driver exhibit several anatomical phenotypes. (B) Giant fibers maintained "wild type" anatomy in nearly 38% of animals tested. C, D, E) Giant fibers lacking terminals were paired with ipsilateral (25%), contralateral (8%), and bilateral (17%) giant fibers. F) In one specimen, both giant fibers were "bendless". n= 24 animals.

Table 3.2. Phenotypic distribution following genetic alterations in the Netrin/Frazzled pathway. Frequencies of each phenotypic outcome are shown for each genotype tested in the Netrin and Frazzled pathway. Frequencies are given in percentages of the number of animals tested in that genotype (given under the "n" column). Dashes within the chart indicate no presence of that phenotype.

Genotype	n															
A307:GAL4; UAS-*netA*	12	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c17:GAL4; UAS-*netA*	10	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ShakB:GAL4; UAS-*netA*	14	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A307:GAL4; UAS-*netB*	12	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c17:GAL4; UAS-*netB*	8	87.5	-	-	-	-	-	-	-	-	-	12.5	-	-	-	-
ShakB:GAL4; UAS-*netB*	8	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NetA Δ	12	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NetB Δ	9	66.7	-	-	-	-	-	-	-	-	-	-	-	22.2	11.1	-
NetA ΔB Δ	8	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NetAΔB-TM	7	14.3	-	-	-	-	-	-	-	-	-	28.6	28.6	-	28.6	-
NetAΔB-sec	9	88.9	-	-	-	-	-	-	-	-	-	1	-	-	-	-
A307:GAL4; UAS-*frazzled* (hetero)	24	37.5	-	-	-	-	-	16.7	25	8.3	4.2	4.2	-	-	-	4.2
A307:GAL4; UAS-*frazzledΔC*	54	75.9	9.3	5.6	3.7	1.9	-	-	-	3.7	-	-	-	-	-	-
A307:GAL4; UAS-*frazzled* (homo)	13	30.8	-	15.4	7.7	7.7	23.1	15.4	-	-	-	-	-	-	-	-

Presynaptic Anatomy	A 	B 		C 		D 		E 
n (animals)	9	6		2		4		1
TTM Latency (ms)	0.92 ±0.12	0.84 ±0.074	2.58 ±0.376	0.82 ±0.261	2.3 ±0.035	1.23 ±0.697	1.59 ±0.424	na
FF-100Hz (%)	90.1	91.7	14	68	7.5	51.8	33.8	na
FF-200Hz (%)	70	72.5	10.7	54.5	12.5	50.8	16.3	na

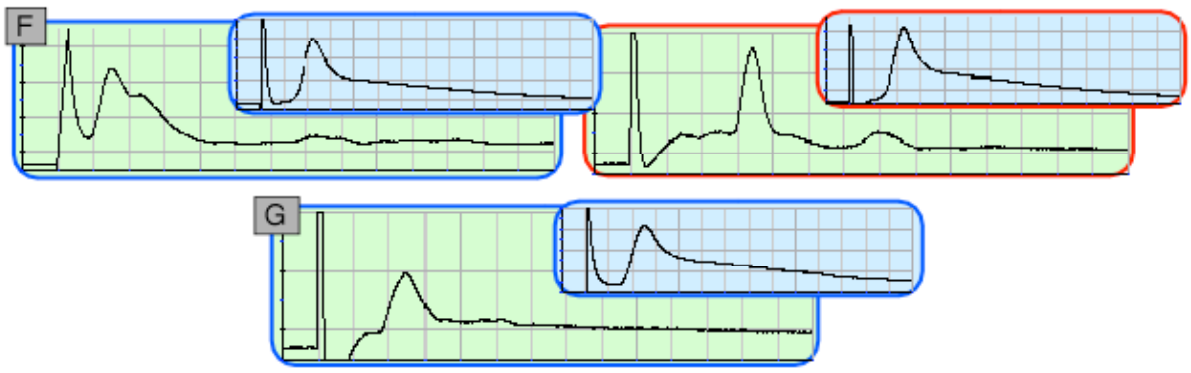


Figure 3.3. Electrophysiological effect of Frazzled overexpression. Latency of response to direct brain stimulation (ms) and following frequency to train stimulation at 100 and 200 Hz are given for each observed anatomical phenotype (cartooned at top). A) Nine of the twenty-two animals showed wild type terminals with normal physiology. B,C) Single-terminal giant fibers paired with a “bendless” axon. Electrophysiology is colored to match the axon whose terminal extended to the recorded side (blue=left axon, red=right axon). D) Bilateral giant fibers paired with a “bendless” axon formed terminals on both left and right TTMns. Recordings from ipsilateral TTMn are shown on left, contralateral on right. E) In a single case, neither giant fiber elaborated a presynaptic terminal to the TTMn. No response was recorded from either TTM (na). F) Representative traces of latency shifts from animals with a single terminal paired with no terminal (as in B and C). The traces on the left (outlined in blue) are from the TTM (green background) and DLM (blue background) driven by the giant fiber with a fully elaborated terminal at the TTMn. The traces on the right (outlined in red) are from the TTM and DLM driven by the other giant fiber (no terminal at TTMn). G) Representative traces from a bilateral giant fiber (TTM and DLM from single animal). Demarcations in TTM traces represent 10mV in Y, 1ms in X. Demarcations in DLM traces represent 20mV in Y, 1ms in X.

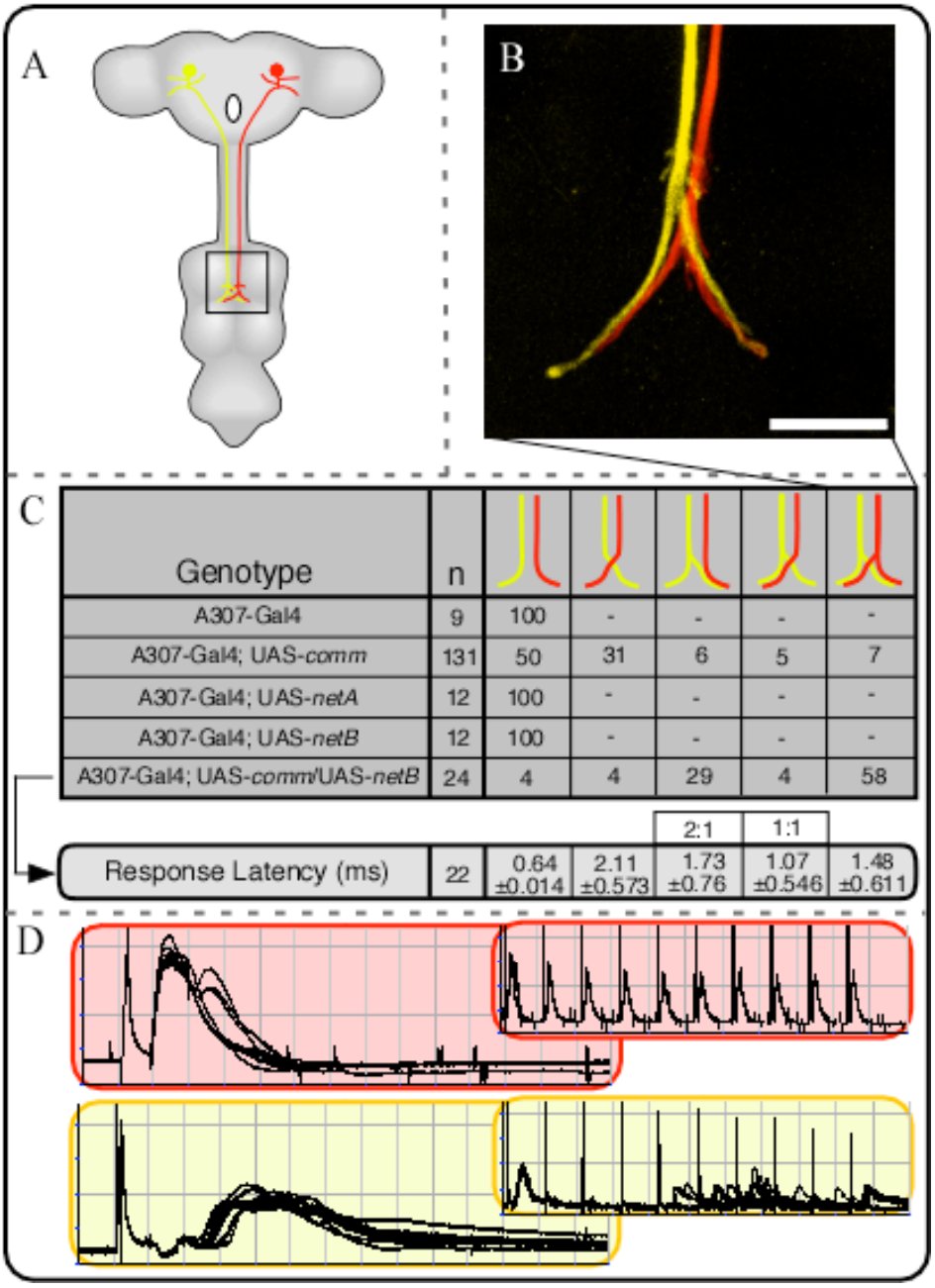


Figure 3.4. Overexpression Netrin in a UAS-Commissureless background removes competitive pressure. A) Cartoon of dissected CNS showing location of imaged terminals. B) Maximum intensity projection of confocal series showing both giant fibers with bilateral terminals (yellow = Lucifer yellow; red = Rhodamine; scale=25μm). C) Phenotypic distributions, compared with previous genotypes (n= animals in each genotype, dashes indicate no phenotypic presence). Electrophysiology data shown for A307-Gal4:UAS-*comm*/UAS-*netB*. Latencies for animals with a bilateral giant fiber paired with a non-bilateral (phenotypes third and forth from the left in the cartoon) are divided into the TTMn hosting two terminals (2:1) and hosting a single branch of the bilateral (1:1). D) Representative electrophysiology trace from an animal with a bilateral terminal (R), paired with an ipsilateral (L), recorded from the TTM following direct brain stimulation (train at 100Hz). Demarcations in Y=20ms, X=1ms/10ms for latency/train

CHAPTER 4

DATA ANALYSIS AND MODEL DEVELOPMENT: THE GIANT FIBER AS A PLASTIC, COMPETITIVE MODEL

4.1 Introduction

The Giant Fiber System provides a unique opportunity to examine the interplay between neurons during synaptogenesis. The bilateral symmetry of homologs meets the defined requirements of a competitively sculpted circuit while existing in a highly tractable invertebrate model system. This offers us a rare chance to study the molecular mechanisms that guide target recognition and competitive synaptogenesis. However, just as in vertebrates, the wild type system gives no indication of competition. Rather, the highly stereotyped and exclusive synapses would indicate non-plastic genetic control. However, by applying an experimental paradigm established by the first observers of neuronal competition, in this case ablation, I was able to reveal the system's inherent plasticity. The removal of a postsynaptic component affected the development on both sides of the synapse in significant and repeatable ways. Postsynaptic elements responded by expanding to fill the vacated territory. Presynaptically, the deprived axon grew across the midline and formed its terminal along intact TTMn, concurrently innervated by the other giant fiber. These adaptations to our perturbation provided insight into the internal dynamics of the Giant Fiber System, namely an inherent plasticity on both sides of the synapse.

In this chapter, I examine the data and develop a model for giant fiber plasticity. First, I review the unilateral ablation experiment specifically and use the results to create a predictive model, one of a plastic if non-competitive system. However, I go on to show that the guidance data lie in contrast to these initial predictions, so the model shifts to one of hidden competition. I modify this new model with results from my experiments with repulsion and attraction, culminating with an intriguing link between the two. Once I have formed my working model for the observed dynamics, I return to each result and describe how the model would produce the observed phenotypes, ending again on the unilateral ablation.

4.2 Responses from both pre- and postsynaptic elements to ablation reveal plasticity.

4.2.1 Effect of unilateral TTMn ablation on the intact TTMn

Morphologically, the most pronounced response to unilateral ablation was the reactive expansion of the dendritic arbor of the remaining TTMn across the midline. The expansion was highly branched and was contacted by both giant fibers, frequently following along axons. The area covered by the intact dendrite expanded noticeably. If the TTMns are a source of a pro-synaptogenic factor, it is possible that the increase in dendritic arbor size may have correlated to an increased production of this factor from the one remaining TTMn, even though the systemic level of pro-synaptogenic factor is reduced by the removal of the other source cell.

4.2.2 Effect of TTMn ablation on giant fiber guidance

Bilateral ablation reveals that in the absence of TTMns, the giant fibers failed to extend a presynaptic terminal posterior to the PSI connection. This supports the existence of a pro-synaptic factor that is both required and attractive to the giant fiber. In unilateral ablations, the target deprived giant fiber crossed the midline in over 90% of animals tested, while the non-deprived giant fiber extends to its target TTMn normally without crossing the midline (Figure 2.4). This implies that the crossed giant fiber is a response to the contralateral TTMn, not just arbitrary growth.

Second, the act of crossing demonstrated an apparent reduction of Slit/Robo signaling within the target deprived giant fiber. This pathway normally prevents giant fibers from crossing the midline in wild type animals (Godenschwege et. al., 2002b). Either there was a reduction in Slit ligand at the midline or a reduction in Roundabout receptor on the axon. If Slit were reduced, we would expect a less localized response perhaps affecting the CNS as a whole, but specifically the non-deprived giant fiber. However, the axon on the non-ablated side remained ipsilateral, ignoring the TTMn dendrites across the midline, indicating that Slit is still being produced by midline glia (Figure 2.4, yellow axon). Taken together, these conclusions support a model where, in response to the loss of an ipsilateral target, a giant fiber underwent a cell-autonomous decrease in Roundabout signaling that allowed it to cross the midline and access the intact TTMn on the other side.

4.2.3 Effect of unilateral TTMn ablation on terminal overlap

In the wild type system, giant fibers remain ipsilateral and connect exclusively to the TTMn on the same side. However, when we removed one TTMn, the two giant fibers both formed their terminal bends along the same cell, often in direct contact with each other. The simplest explanation for this single result is that the exclusivity observed in wild type animals is entirely a result of midline repulsion by Robo/Slit; giant fibers are naturally capable of growing toward either one or both TTMns, regardless of concurrent innervation from another axon. Removing, or overcoming this barrier would not only allow crossing, but would also result in overlapping presynaptic terminals. If we assume that repulsion from the midline is the only thing limiting a giant fiber from contacting the contralateral TTMn, we can predict possible outcomes in its absence.

4.3 Considerations from the Unilateral Ablation

4.3.1 Repulsion provides exclusivity

Using the results and model described above, we can predict possible terminal morphology outcomes in an environment lacking a repulsive force at the midline. These possibilities, however, will be influenced by what is more developmentally pressing for the giant fiber – to occupy the most synaptic territory or form the most efficient synapse. If we assume (1) a territory-driven development, giant fibers would be expected to form primarily bilateral synapses, with each TTMn

receiving input from both fibers (Figure 4.1B). This is certainly possible, as we have observed the ability of a single axon to form bilateral connections in several genetic backgrounds and ablation studies (Table 4.1, purple column). Alternatively, if we assume (2) a synaptic strength-driven development, we would expect each giant fiber to contact a single TTMn (Figure 4.1C). This too is possible, since expansion of a synaptic terminal has been shown to compromise the efficiency of an individual synapse (Figure 3.3D; Murphey & Lemere, 1984 [cartooned in Figure 1.3A, panel II]). If each giant fiber forms a single terminal, the TTMn chosen would be random, and we would expect a pattern of outcomes similar to flipping two coins simultaneously (Figure 4.1C). In 25% of cases, each axon would synapse with its ipsilateral TTMn (heads, heads). In another 25%, both axons would synapse with the contralateral TTMn (tails, tails). In the remaining 50% of the animals, we could expect that one giant fiber will stay ipsilateral while the other synapses contralaterally, resulting in overlapping terminals on one TTMn and no connections on the other (heads, tails). However, the results following reduction of the midline repulsion do not follow either of these predicted outcomes, forcing us to adapt our model.

4.3.2 Exclusivity without repulsion

Expression of *commissureless*, the downregulator of Roundabout, was shown to allow the giant fiber axons to cross the midline. This occurred at near random distributions, with 53% staying on their ipsilateral side and 47% crossing the midline

(Table 3.1, Table 4.1, Figure 4.2 – contralateral and bilateral axons scored as midline crossers). This supported the assumption that giant fibers have the native ability to form contralateral terminals – prevented by midline repulsion. However, despite the ability for axons to cross with random frequency, axons retained a distribution of one axon per TTMn (as seen in the wild type system) in 87% of the animals (Figure 4.3). The exclusivity was maintained whether both axons formed ipsilateral or contralateral lateral bends. In fact, even among animals where one giant fiber formed a bilateral terminal, the other axon was twice as likely to form a unilateral terminal rather than forming a bilateral itself.

The results from the *commissureless* over-expression studies show that while midline repulsion could be readily muted, this did not necessarily lead to a loss of exclusivity as predicted by our earlier model. Therefore, we must conclude that these two behaviors of the giant fiber are not outputs of the same pathway. Rather, there appears to be a second pathway, separate from sensitivity to midline repulsion, which prevented two giant fibers from forming terminals on the same TTMn. While both TTMn were equally attractive to both axons – both giant fibers were capable of growing toward either – this second pathway acted to limit the input of each dendrite to a single axon. Interestingly, the same signaling pathway that is suspected to play an attractive role may hold the key to giant fiber plasticity and synaptic redundancy.

4.4 A Connection Between Attraction and Repulsion

The indirect silencing of Roundabout resolved two pathways from the ablation result: one regulating repulsion of the axon from the midline, the other regulating the giant fibers attractive response to a potentially occupied target. This introduced the idea that there may be an attractive pathway involved in the giant fibers' exclusive connectivity. When we over-express the attractive receptor, Frazzled, in the giant fiber system, however, we saw alterations in not just exclusivity, but midline crossing as well. Specifically, giant fibers were seen to cross the midline in just over half the animals examined (57%, n=26: Table 4.1, A307-Gal4; UAS-*frazzled* (homo); Figure 4.2). Again, it was possible that an increase in attractive receptors simply shifted the balance of attraction and repulsion signals within the axon. However, if increasing this pathway of canonical attraction was an effective means to induce crossing, we would expect overexpression of Netrin to have a similar outcome. We observe no such effect when over-expressing *netrin* in the Giant Fiber System (Figure 4.2, A307-Gal4; UAS-*netrinB*). Alternatively, another explanation is that the receptor, Frazzled, is acting outside its canonical attractive role to affect repulsion.

Recent work has presented an interesting solution for this mechanistic linchpin. Yang et. al. have shown that Frazzled is able to directly activate the transcription of *commissureless* in a pathway separate from its attractive role (2009). Moreover, this second function for this Netrin receptor appears to do so independently of its native ligand, as the effect is observed in Netrin loss of function

mutants as well. Using a *Drosophila* embryonic model, they demonstrated that Frazzled mutants have lower *commissureless* mRNA levels in eagle neurons, which normally have high levels during the stage of development when they cross the midline. This was shown to be a cell-autonomous effect, as expression of a Frazzled construct in this neuron subset both restores *comm* mRNA levels and rescues the Frazzled mutant phenotype. Furthermore, this Frazzled construct was expressed in apterous (Ap) neurons, which do not express *commissureless* normally and do not cross the midline. Exogenous frazzled expression was shown to not only induce *comm* mRNA in these neurons but also led to midline crossing in 35% of animals tested (Yang et. al, 2009). They concluded that their experiments demonstrate that Frazzled is both necessary and sufficient for expression of *commissureless* mRNA.

This work is strong evidence that the receptor Frazzled performs two tasks in developing neurons, one as a receptor to the attractant Netrin and another in a pathway to silence Roundabout through Commissureless (Figure 4.4). Intriguingly, this second pathway does not require the binding of Netrin, functioning in the absence of this attractant. This role of the "empty receptor" would, at its most basic, explain the similarities between *commissureless* and *frazzled* over-expression (Figure 4.2, 4.3). However, the implications become more impressive when added to the developing model. Frazzled would lie uniquely between two opposing pathways, attraction and repulsion. On one side, Frazzled acts in its canonical function, binding Netrin and signaling positive chemotaxis (Figure 4.4, left panel).

Hypothetically, this attractive function leads to the synaptogenesis between TTMn and the giant fibers, but at its very least it may serve to bring synaptic components to a rendezvous point. This function is usually restricted spatially, with Frazzled only having access to the Netrin on the ipsilateral side due to Roundabout's simultaneous binding of Slit (Figure 4.5A). In wild type animals, this is the primary function of Frazzled, due to a pre-supposed Netrin supply. However, the newly discovered second face of Frazzled would be activated not by its binding to its attractive ligand but by the lack of a binding partner. In situations where there is a significant shift in the Netrin:Frazzled ratio, like the removal of a ligand point source, most available Frazzled remains in an empty state. This shifts the response of Frazzled to its second pathway, *commissureless* induction (Figure 4.4, middle panel; Figure 4.5B). A giant fiber in this situation would then be able to respond to the absence of a ligand source by downregulating its response to midline Slit. This would allow a neuron without a useable or present target to cross the midline in search of a viable replacement. The bilateral ablations add to this, showing that in the absence of both targets, the giant fibers do not elaborate terminals – reiterating that this growth is directed toward the TTMn dendrites.

4.5 Netrin as a Postsynaptic Attractant

The ability of the giant fiber to respond to the loss of its target has led to the incorporation of a molecular switch in our model, one that can activate a secondary signaling pathway in the absence of a primary one. The Frazzled receptor fits nicely

as this switch, as it has been shown to deafen a cell to Slit through induction of *commissureless* expression. But this addition to the model has broader implications. By including Frazzled, the model must take into consideration a role for Netrin in giant fiber dynamics. The logical placement for Netrin would be as the hypothesized postsynaptic attractant. In this role, Netrin would be produced by the TTMn and recognized by Frazzled on the giant fiber (Figure 4.5).

The addition of Netrin to the model allows us to examine other key aspects of giant fiber behavior. For instance, if Netrin is a factor to which both axons respond equally, it may be involved in their competitive selection of targets. Competition relies on some resource, limited in supply and vital to the eventual securing of a dominant connection by the winning competitor. If this signal is produced at a steady and specific level, it is reasonable to conceive a model where a limited supply of Netrin is the driving force behind giant fiber competition. Giant fibers expressing a constant and similar level of Frazzled would subsequently be able to bind and respond to a set level of Netrin. This creates a balance where the Netrin produced by the postsynaptic cells is enough to support one and only one giant fiber based on its Frazzled receptors levels. Once a single axon has bound the available Netrin from a target, there is too low a level remaining to support an additional axon. This balance between “supply and demand” would be the key to competition between giant fibers. A shift in one direction or the other should have implications in giant fiber terminal formation. Theoretically, if Netrin was no longer a limiting factor, we should observe a drop in competitively sculpted phenotypes. In other words, if there

were enough Netrin produced by either TTMn to bind a significant number of Frazzled receptors on both giant fibers, those axons would have no feedback from an “occupied” target. Each giant fiber would be effectively faced with two open targets, equally capable of hosting a terminal, regardless of another incoming axon.

However, unlike repulsion reduction, simply ramping up the attraction ligand was not enough to cause midline crossing, shared targets, or even missing terminals. In fact, except for a single axon that showed minor overgrowth, all morphologies were wild type. The fully functioning midline barrier in these overexpression studies appeared to prevent any contralateral growth. Stuck on its own side, an axon would have had access to plenty of Netrin, meaning the Frazzled would likely not engage the *commissureless* induction pathway. To observe the true effect of Netrin over-abundance, I turned to the combined expression of UAS-*netrin* and UAS-*commissureless*.

4.6 Netrin Relieves Competition in Commissureless Background

The combination of Netrin and Commissureless overexpression in the Giant Fiber System yielded a phenotype unique from overexpression of either alone. Netrin alone had no observable effect on giant fiber structure or function. Commissureless alone was shown to produce an effective blockade of midline repulsion but giant fiber terminals remained exclusive to one TTMn. The combined phenotype, however, maintains the midline insensitivity seen from Commissureless

but with a pronounced increase in the number of shared targets. Just as the Commissureless phenotype without Netrin was interpreted as demonstrating the existence of selective pressure (1:1 despite crossing), the shift in phenotype following the addition of Netrin demonstrates the absence of this selective pressure (2:1, combined with crossing). In other words, there is a correlation between the level of Netrin and an axon's ability to form a synapse on an occupied dendrite – observable only in conjunction with the suppression of midline repulsion.

4.7 A Model for Giant Fiber Targeting, Plasticity, and Competition

The revised model presents a conditional connection between two pathways, repulsion from the midline and attraction to a postsynaptic target. To clarify how this connection is proposed to work, I will present how the model describes each perturbation from wild type, through the alteration of guidance cues, and finishing in the unilateral ablation experiment.

4.7.1 Wild Type phenotype

The most important mechanism in the wild type animal is midline repulsion mediated by the receptor Roundabout and its ligand Slit (Godenschwege et. al., 2002b). Slit/Roundabout signaling prevents the giant fibers from crossing the midline. In the presence of an intact TTMn, releasing normal levels of the Netrin attractant, Frazzled receptors on the giant fiber signal attraction and lead the giant

fibers to the TTMn for synaptogenesis. This is cartooned in Figure 4.5A, on a molecular scale and in Figure 4.6A on a cellular scale (left axon). Dendrites from each TTMn grow medially until they reach the homologous dendrites from the opposite side at the midline. The circuit forms with little to no presynaptic plasticity required, so none is observed (Figure 4.3). There is no midline crossing, and all giant fibers are led into 1:1 connections. (Figure 4.2; Figure 4.6A).

4.7.2 Reduction of Slit/Roundabout midline repulsion

Reduction of Roundabout signaling allowed us to observe underlying competitive mechanisms. Direct over-expression of *commissureless* allowed giant fibers to cross at random levels (Figure 4.6B). However, the targets on both sides were still producing only the normal amount of Netrin, which signaled attraction through Frazzled, but was not high enough to support the innervation of a single TTMn by two giant fibers. This led to the maintenance of the 1:1 connection in 87% of animals (Figure 4.3). This demonstrated a change in one pathway (midline repulsion), while the other pathway (competition) remained fully functional (Figure 4.6B)

4.7.3 Over-expression of Netrin

The result of over-expressing only *netrin A* or *netrin B* was indistinguishable from wild type (Table 4.1; Figure 4.6C). In this condition, all Frazzled receptors were bound with Netrin, leaving no empty receptors to induce *commissureless*. Without

Commissureless to downregulate Robo/Slit signaling, giant fibers were unable to cross the midline. (Figure 4.2; 4.6C). Consequently, since two giant fibers could not access the same target, we were unable to see the result of their interactions, presumed to be non-competitive under these conditions. Instead, axons remained in a 1:1 ratio with dendrites (Figure 4.3). It is only when we re-examined this Netrin fluctuation in a system free of midline restriction, due to overexpression of *commissureless*, that we observed changes in these competitive interactions (Figure 3.4; Figure 4.6D). Frazzled receptors on both giant fibers were fully saturated by plentiful Netrin, meaning they neither signal for *commissureless* production nor provided the drive for competitive synaptogenesis (Figure 4.6D). The result was a giant fiber system with almost no competitive pressure, leading to giant fibers that freely crossed the midline and formed overlapping, bilateral synapses (Figure 3.4; Figure 4.6E).

4.7.4 Over-expression of Frazzled

An overabundance of Frazzled translated to an increase in empty receptors. By our model, these empty receptors induced *commissureless*, indirectly silencing Roundabout signaling (Figure 4.4; Figure 4.5B; Figure 4.6E). This created a situation where midline crossing increased proportionally to the amount of excess Frazzled (Figure 4.2). Additionally, Netrin produced by the TTMn remained constant, maintaining a competitive pressure, demonstrated in the large majority of connections forming in a 1:1 ratio (Figure 4.3, 77% for single dose).

Frazzled's "empty receptor" pathway was also able to be induced through the expression of the putative dominant negative, FrazzledΔC. This construct is capable of binding Netrin, but lacks the intracellular components to signal either attraction or commissureless induction. In this capacity, it can be thought of as a sink for Netrin, leaving less ligand available for endogenous, fully functional Frazzled. The phenotype had low penetrance, but the trend was still present. Axons were able to cross the midline (16%, Figure 4.2), while exclusivity of the connections remains (86% 1:1, Figure 4.3). It should be noted that if Frazzled has this second function in this system, expression of a putative Netrin sink would not be considered a classic dominant negative experiment, but rather a means to shift relative levels of ligand and receptor.

4.7.5 A second look at dose dependence

The *frazzled* over-expression studies presented an interesting subtlety in the dynamics captured by this model. Unlike expression of two copies, a single copy of the UAS-*frazzled* transgene resulted in a synaptic reduction with nearly 30% of giant fibers failing to elaborate a synaptic terminal beyond the PSI, in a "bendless"-like phenotype. However, in nearly half of these cases, the lack of synaptic terminal of one giant fiber was paired with territorial invasion by the other giant fiber (Table 3.2). Interestingly, the crossing of invading axons into unoccupied territory accounted for all but one case of midline crossing. So while there was a novel decrease in overall connectivity, the system maintained exclusivity in nearly 80% of the terminals

present (Figure 4.3). Additionally, while invading axons crossing the midline accounts for 14% of animals observed, this is well below the 50% seen in *commissureless* over-expression studies, suggesting that a single dose of the *frazzled* may have induced *commissureless* expression, but not to the level of Gal-4 driven expression. Doubling the dose of Frazzled, however, did result in a midline-crossing rate of 57%, supporting a mechanism where by excess (or empty) Frazzled triggers *commissureless* at sufficient levels to allow free crossing (Table 4.1; Figure 4.2 (A307-Gal4; UAS-*frazzled* (homo)). Despite the lower than expected crossing rate with a single dose, the response of axons to their homolog's absence at the TTMn does shed light on some important dynamics. Consider the invasion of one axon into another's territory. Invasion occurred when the territory is unoccupied by another axon, implying that competition may still have been restricting axons from forming overlapping connections. However, mere absence of a competitor did not appear to be adequate stimulus to draw an axon across the midline. In fact, only half of unoccupied TTMn's receive input from the contralateral axon (Figure 3.3B-E). In other words, under these expression conditions, target availability is necessary, but not sufficient for midline crossing of an axon, from a competitive standpoint. This is in direct contrast to the ablation conditions in which the presence of another target (and the absence of the other) was sufficient to draw an axon across, but its (competitive) availability was unnecessary. This again suggests that Frazzled may be at the heart of this competitive switch, playing a key role in determining when and how an axon responds to its brother across the midline.

4.7.6 Unilateral Ablation of a TTMn

Finally, we come to the unilateral TTMn ablation experiment. In this scenario, the two giant fibers developed under markedly different conditions. One arrived to its target to find a local source of Netrin, while the other found nothing beyond the PSI (Figure 4.5). The giant fiber with an intact target developed with both canonical inputs, Roundabout and Slit for midline repulsion, Frazzled and Netrin for guidance to the target (Figure 4.5A, left axon; Figure 4.6A,). Accordingly, this giant fiber never crossed the midline. It's homolog however, experienced a different environment. Upon arriving to the synaptic region, posterior to the PSI, the giant fiber was met by no local source of Netrin (Figure 4.5B, right axon; Figure 4.6E). Frazzled on its surface remained unoccupied and in turn would have induced *commissureless* expression. Once Roundabout had been silenced, these axons were free to cross the midline, which they did at a rate of over 90% (Figure 4.2). Interestingly, the competitive force that would have normally prevented both axons from forming simultaneous terminals on this dendrite appears to be absent. In fact, every example of an orphaned giant fiber crossing the midline resulted in an overlapped synapse. There are two possible explanations for this lack of competition. Either the target's production of Netrin has increased (possibly in proportion to its increased size), or there has been a change in the orphaned axon, in addition to the induced *commissureless* expression, that alters the normal competitive mechanism.

4.8 Implications and conclusions

During the analysis of the data described here, much time was spent to understand the phenomenon and mechanism that allows a giant fiber, orphaned or otherwise manipulated, to cross the midline. However, the more important questions lie in what happens when it gets there. Here I have shown that it is not the fact that giant fibers are able to cross (which occurs through a unique and incompletely characterized pathway) that is most pertinent, but that this crossing remains subject to competitive laws. I propose that these laws were governed, in part, by the same attractive forces that guide the giant fiber to its final target. Interestingly, the data presented here suggest a complexity to this signaling previously not considered. Opposing canonical guidance pathways appear linked and readily altered by giant fibers according to an apparent hierarchy. Additionally, giant fibers seem subject to an overriding synaptic requirement, even when it comes at the expense of competitive mechanisms and even synaptic function.

The proposed model is one of pathway interconnections, signal balancing, plastic synaptogenesis, and regulated territory management. It is the first step on the road to understanding the complex forces that an axon must assess and integrate to both find its unique target and form an exclusive connection to it. The work in this dissertation breaks ground for further study into competitive dynamics by establishing the Giant Fiber System as a circuit capable of triggered plasticity. Interestingly, this plasticity does not currently appear to play an active role in the

wiring of the wild type circuit. Rather it seems to exist below the surface, ensuring that axons possess the capability to adapt to circuit defects and errors. It is worth reflecting that the original aim was not necessarily to characterize a naturally engaged competitive system, of which there are several being studied. Rather, to uncover and dissect the raw molecular machinery underneath a tractable model. The plasticity discovered within the Giant Fibers System opens the door for us in the larger goal of studying competition. By being purposefully reductive in the circuits examined we build a framework within which to characterize the enigma of competition. I submit this work in support of the Giant Fiber Reflex as that framework.

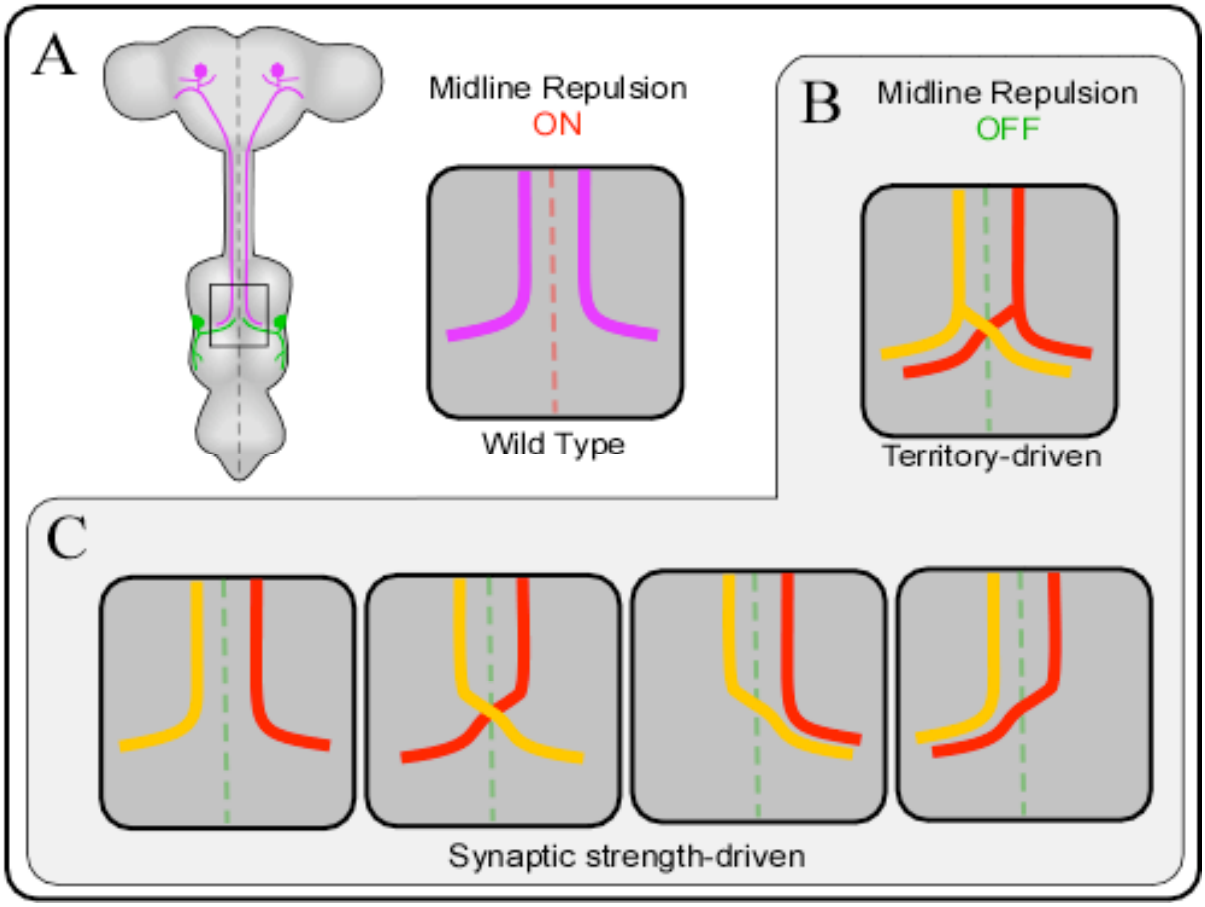
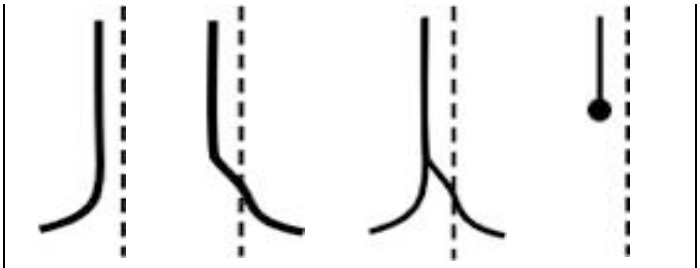


Figure 4.1. Predicted morphological outcomes using the "repulsion only" model. A) The giant fibers of the wild type system remain ipsilateral due to the midline barrier (red dashed line) provided by Slit/Roundabout signaling. B, C) In the absence of the midline repuslion (green dashed line), axons are predicted to demonstrate an unrestricted side "choice". B) Giant axons are primarily predicted to grow bilaterally if the driving force is expansion of the largest possible terminal territory. C) Assuming axons are pressured to form terminals to single TTMns, on either side at random, four outcomes result from non-biased, random distribution. This last prediction assumes each giant fiber has free access to either side, with no pressure outside the absent repulsion restricting the outcome.

Table 4.1. Individual giant fiber morphologies in different backgrounds. The chart displays the frequency of a giant fiber in each genotype to form a terminal to one side or another. Giant fibers that formed terminals on the ipsilateral side are shown in the yellow column, while giant fibers that formed terminals on only the contralateral TTMn are shown in the pink column. Bilateral terminals (those formed on both TTMn) are shown in the purple column, but still demonstrated an ability to cross the midline. Giant fibers that did not form any presynaptic terminal beyond the PSI are shown in the grey column and labeled "bendless". Cartoons at the top of each column illustrate the listed axons' morphologies in relation to the midline. NR=not reported**Godenschwege et. al. (2002b)



Genotype	n (GF)	Ipsilateral (%)	Contralateral (%)	Bilateral (%)	"Bendless" (%)
A307-Gal4; +	18	100	0	0	0
A307-Gal4; UAS-comm	262	53	34	13	0
A307-Gal4; UAS-roboΔC**	22	NR	27	NR	5
A307-Gal4; UAS-a-robo RNAi	13	54	23	15	8
Ablation (target-deprived)	21	0	55	36	9
Ablation (target-intact)	21	100	0	0	0
A307-Gal4; UAS-frazzled (hetero)	48	56	4	10	29
A307-Gal4; UAS-frazzledΔC	108	82	13	3	2
A307-Gal4; UAS-netA	24	100	0	0	0
A307-Gal4; UAS-netB	24	100	0	0	0
A307-Gal4; UAS-comm/UAS-netB	48	19	6	75	0
A307-Gal4; UAS-frazzled (homo)	26	35	19	38	8

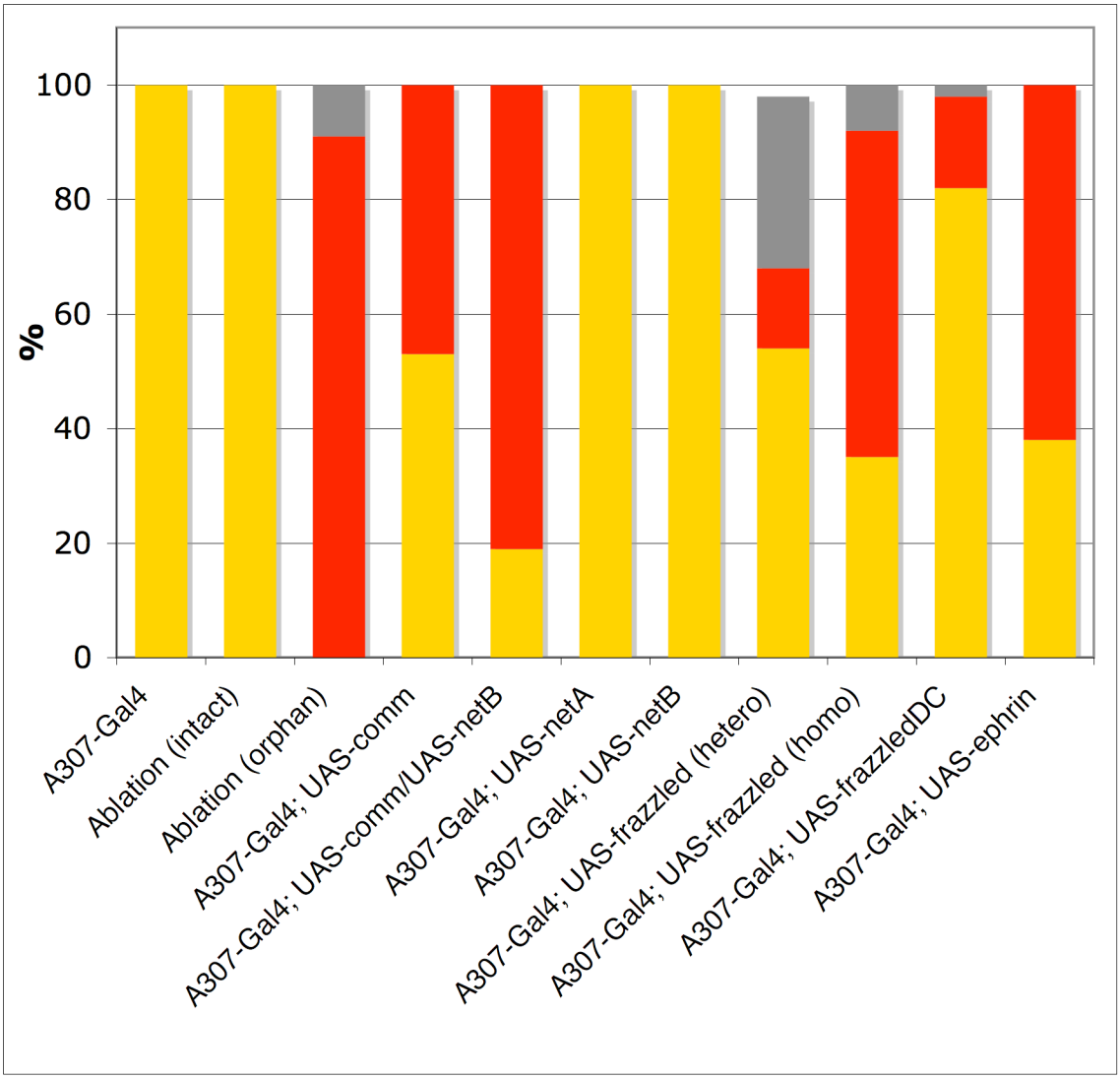


Figure 4.2. Frequencies of midline crossing by the giant fibers in different genetic backgrounds. Giant fibers that formed a single terminal to the ipsilateral giant fiber are represented in **YELLOW**. Axons that crossed the midline are represented in **RED**. This category includes both contralateral and bilateral terminals. Axons that failed to elaborate a terminal are represented in **GREY**. Scores were normalized to the total number of giant fibers in each background and displayed as a percentage of the whole.

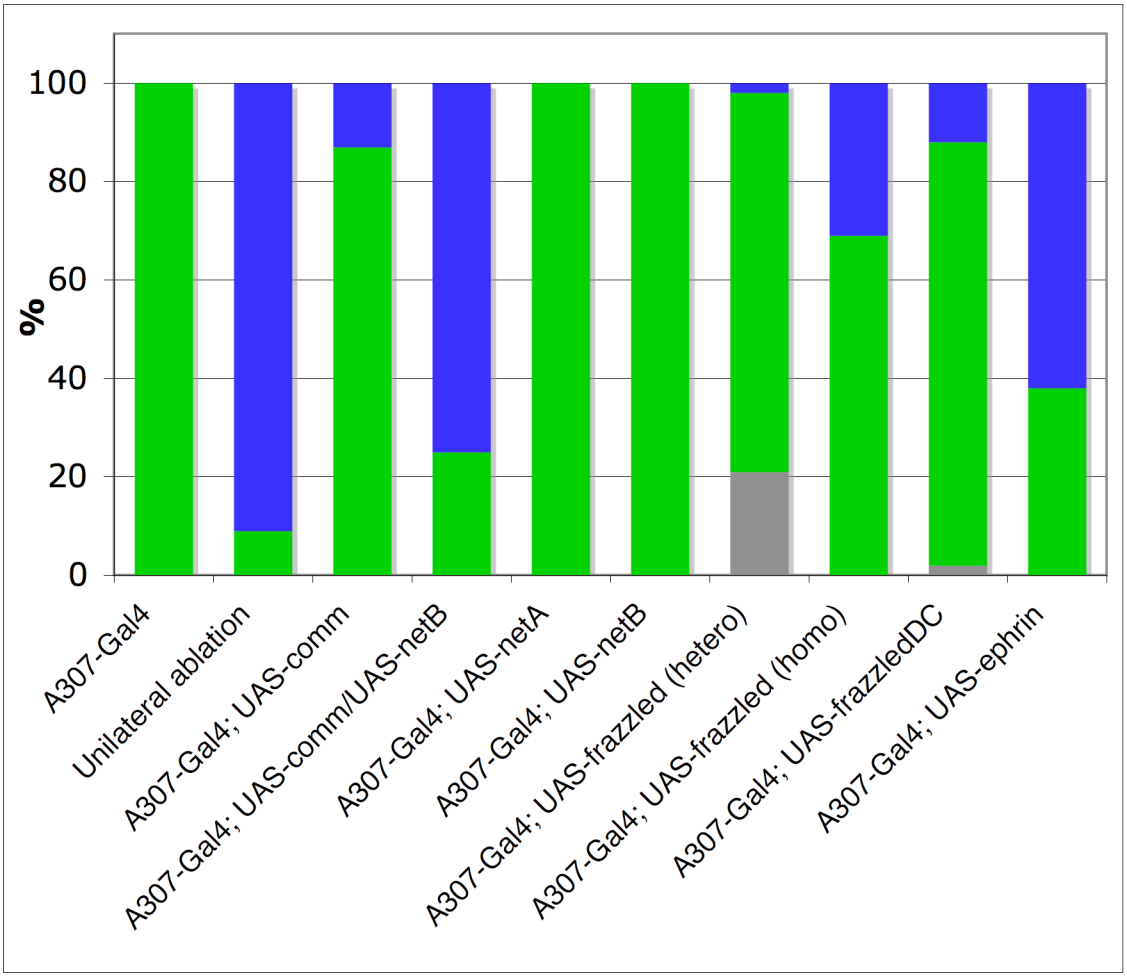


Figure 4.3. The innervation overlap of the giant fibers on a single TTMn in different genetic backgrounds. TTMns with a single giant fiber terminal are represented in **GREEN** **(1:1)**. TTMns with terminals from both giant fibers are represented in **BLUE** **(2:1)**. TTMns that had no giant fiber terminals are represented in **GREY** **(0:1)**. Scorings were normalized to total TTMn in each background and shown as a percentage of the whole.

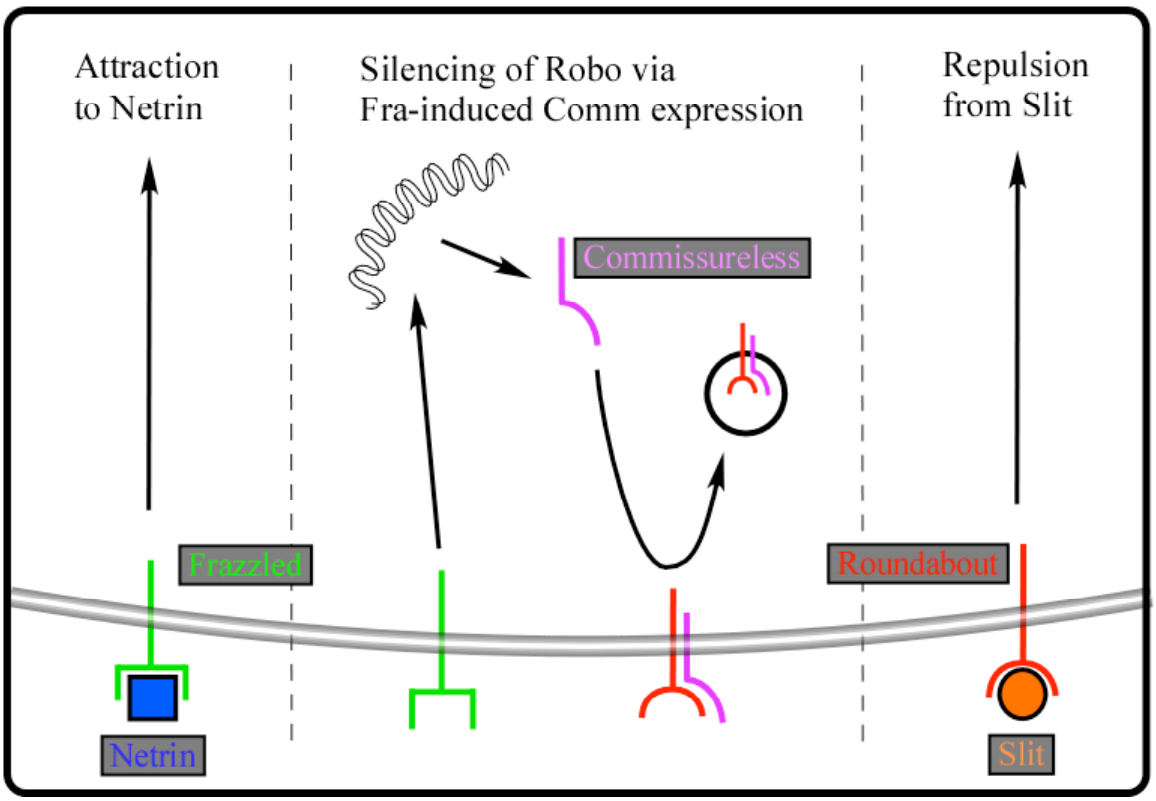


Figure 4.4. Separate attractive and repulsive pathways are connected through a second identified Frazzled pathway. When bound to Netrin (blue), Frazzled (green) signals attractive chemotaxis in growth cones through the canonical pathway. Independent of its Netrin binding, Frazzled can stimulate the expression of Commissureless (purple). Commissureless acts to remove Roundabout (red) from growth cones. In the absence of Commissureless, Roundabout is expressed on the surface and binds Slit (orange), signaling repulsive chemotaxis through the canonical pathway.

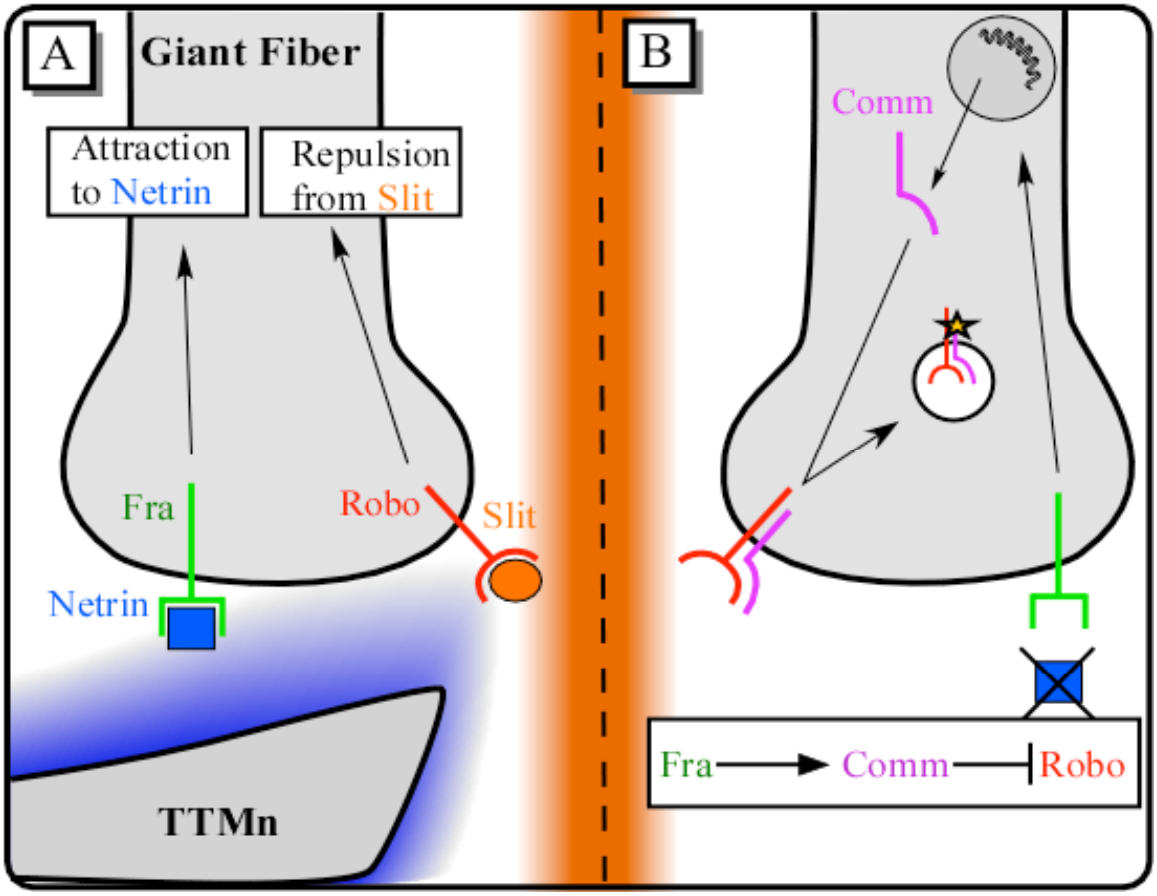


Figure 4.5. Signaling interactions within the giant fiber in different environments. (A) In the presence of canonical ligand, Netrin (blue), Frazzled (green) signals attractive growth. In the presence of its ligand, Slit (orange), Roundabout (red) signals repulsion away from the midline. (B) In the absence of a ligand, Frazzled initiates production of Commissureless (purple). Commissureless removes Roundabout, preventing it translating the Slit midline signal into repulsion.

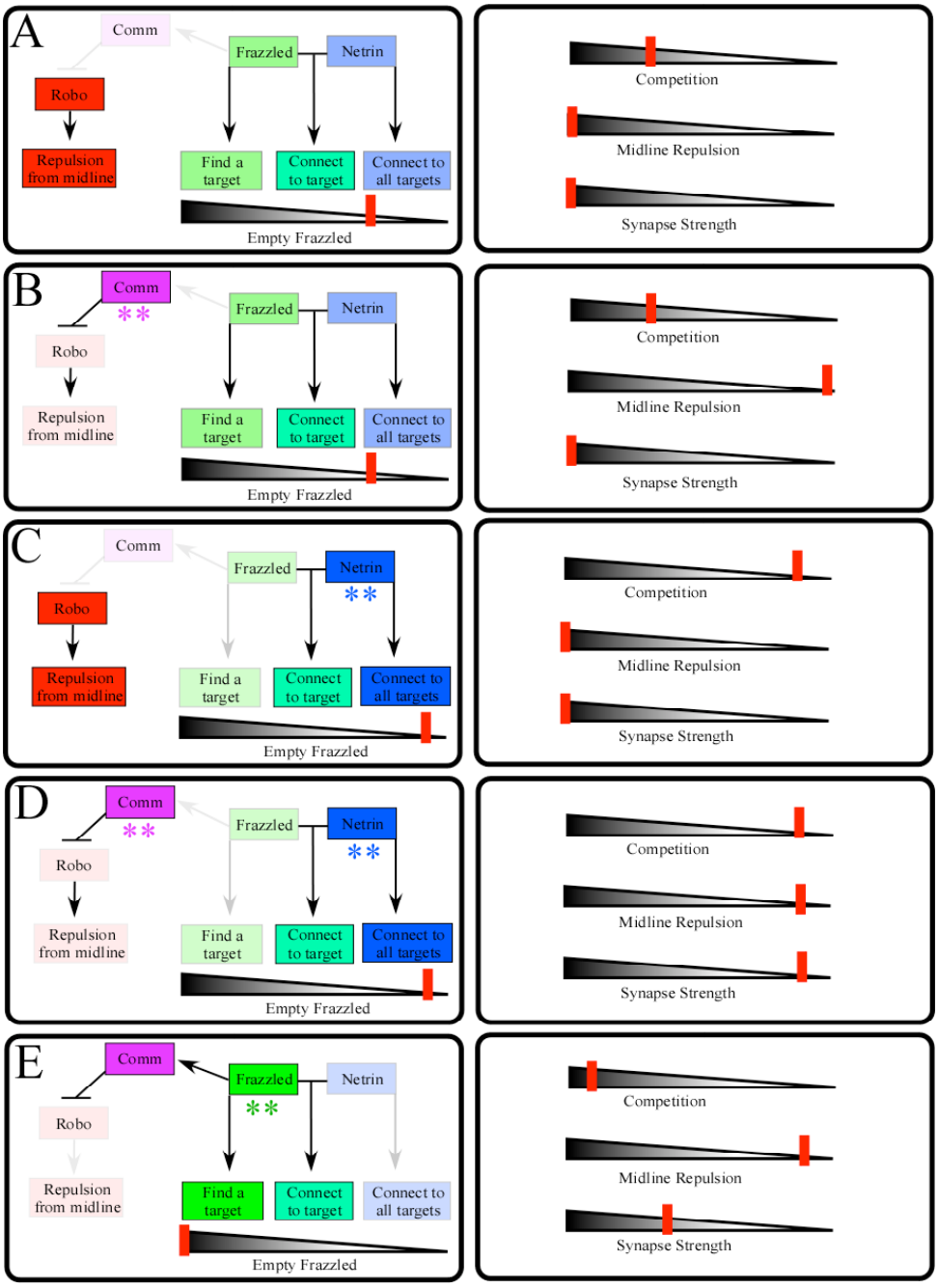


Figure 4.6. Pathway models and signaling changes under different conditions. A)The wild type signal from and Slit/Roundabout provides midline repulsion and Netrin levels keep Frazzled receptors relatively occupied. B) Addition of Commissureless reduced Slit/Robo midline repulsion, but leaves attraction and competition at wild type levels. C) Over-expression of *netrin* reduces the number of unoccupied Frazzled, but Slit/Robo blocks any midline crossing. D) Removal of Slit/Robo signals with Commissureless allows for observation of the Netrin-flooded system. E) Increasing the Frazzled:Netrin ratio – through Fra over-expression or Netrin source removal activates the receptor's ability to induce Commissureless production. ** Gal4 driven over-expression.

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